AN INVESTIGATION ON THE OCCURRENCE AND SIGNIFICANCE OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE IN PHYTOPLANKTON AND NATURAL AQUATIC COMMUNITIES

by

David Alex Francko

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1980

DISCLAIMER

ABSTRACT

AN INVESTIGATION ON THE OCCURRENCE AND SIGNIFICANCE OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE IN PHYTOPLANKTON AND NATURAL AQUATIC COMMUNITIES

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This study demonstrates, on the basis of several analytical criteria, that the production and extracellular release of cyclic adenosine 3':5'-monophosphate (cAMP) is widespread among phytoplankton species. The production and release of cAMP varied markedly among different species grown under similar environmental conditions, and intraspecifically during the life cycle of a given algal species.

In the blue-green alga Anabaena flos-aquae, the collective evidence indicated that cAMP may be involved in the regulation of primary productivity, chlorophyll a synthesis, and nutrient dynamics. In stationary phase cultures of Anabaena, the concentration of cellular cAMP in cells grown under a variety of nutrient regimes was significantly correlated to ^{14}C-bicarbonate uptake. Nitrate-limitation in actively growing Anabaena resulted in increased cellular cAMP production, preceding heterocyst formation. Phosphate-limitation resulted in reduced extracellular release of cAMP, but cellular cAMP was not correlated to the induction of alkaline phosphatase. The addition of the methylxanthine MIX to actively growing Anabaena resulted in increased levels of extracellular cAMP, indicating that Anabaena has a methylxanthine-sensitive cyclic nucleotide phosphodi-

esterase, the first time such an enzyme has been found in a prokaryotic organism. MIX-induced increases in extracellular cAMP resulted in increased chlorophyll *a* synthesis and growth rates compared to control cultures grown without MIX. The addition of large amounts of crystalline cAMP to *Anabaena* cultures supported the view that high extracellular cAMP levels may be involved in chlorophyll *a* regulation, and provided preliminary evidence that the natural turnover rate of extracellular cAMP in cultured *Anabaena* may be very slow. The addition of crystalline cyclic 3':5'-nucleotide phosphodiesterase to *Anabaena* cultures did not result in hydrolysis of extracellular cAMP, suggesting that *Anabaena* may produce an inhibitor of this enzyme, although proteolytic degredation of added phosphodiesterase could not be discounted.

Large differences were noted in cellular and extracellular cAMP production in *Anabaena* during active growth phases and in stationary phase, irrespective of nutrient availability, suggesting that cAMP may be involved in the synchronization of the cell cycle in this organism. In stationary phase cultures of *Anabaena*, the majority of cAMP was found in the extracellular phase, while in actively-growing cultures, much of the cAMP noted was associated with cells.

This investigation marks the first time cAMP has been investigated in natural aquatic systems. An examination of epilimnetic lakewater samples from Lawrence Lake, a hardwater oligotrophic lake, and Wintergreen Lake, a hardwater hypereutrophic lake, both in southwestern Michigan, demonstrated that cAMP existed in both particulate-associated and dissolved forms in these systems. The epilimnetic concentrations of both particulate and dissolved cAMP varied greatly seasonally and between the lakes. Much more cAMP was found in the dissolved phase than the particulate phase on a per liter basis in both lakes, and the rate of cAMP release by epilimnetic

plankton was much higher than that noted in studies on cultured phytoplankton. Particulate cAMP was largely associated with phytoplankton in both lakes. Dissolved cAMP may have resulted from release by epilimnetic plankton and inputs to the pelagial zone from littoral and allocthonous sources.

The dynamics of cAMP in these systems could be correlated to the dynamics of alkaline phosphatase activity and chlorophyll a synthesis during the development of certain phytoplankton associations, but not on a seasonal basis. In Lawrence Lake, particulate cAMP levels could be correlated to the in situ rate of epilimnetic primary productivity on a seasonal basis. In both lakes, cAMP dynamics were closely associated with changes in phytoplanktonic population densities and changes in the species composition of the epilimnetic phytoplankton community.

Large seasonal and interlake differences were noted in the levels of particulate and dissolved cAMP in littoral and pelagial samples collected from both lakes. Examination of two species of aquatic macrophytes common to these systems, Potamogeton zosteriformis and Ceratophyllum demersum, indicated that macrophytic production of cAMP may be important in these systems.

Collectively, data on these trophically dissimilar lakes indicate that cAMP may be involved in the regulation of algal homeostatic responses to changing environmental conditions, particularly in the regulation of nutrient dynamic behavior and changes in the rate of primary productivity. Further, cAMP may be directly involved in mechanisms which regulate the natural rates of increase of phytoplanktonic organisms.

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To Diana

ACKNOWLEDGEMENTS

I am deeply grateful to my major professor, Dr. Robert G. Wetzel for his advice, support, and encouragement during the course of this investigation. Special thanks are also extended to Drs. Philip Filner, Pat Werner, and Peter Murphy for their assistance on my committee. A special measure of appreciation is extended to Drs. Philip Filner, Ray Bressan, and Avtar Handa for their advice on experimental design, without which this project could not have been done. I would also like to acknowledge the technical help of Jay Sonnad and the graphical assistance of Anita Johnson.

My appreciation is extended to Drs. Robert Heath, Jim Grace, and Sven Beer and Art Stewart, Bill Crumpton, Joyce Dickerman, and Steve Weiss for their comments on this investigation.

I am deeply indebted to my wife, Diana, whose continued love and friendship have made this the happiest period of my life. Thanks are also extended to my parents for their continued love and encouragement.

This work was supported by the Office of Water Resources Research (A-103-MICH) and the U. S. Department of Energy (EY-76-S-02-1599, COO-1599-154).

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INTRODUCTION

Although it is known that phytoplankton modify their metabolic responses to changing environmental conditions, little is known of the molecular mechanisms controlling these changes. For example, it is known that phosphate limitation induces the production of alkaline phosphatase in many cultured algal species (Fitzgerald and Nelson, 1966). Similarly, some blue-green algae are capable of heterocyst formation in response to limitation of combined nitrogen (Wolk, 1973). The dynamic nature of chlorophyll synthesis, primary productivity, and growth rates during the life cycles of photosynthetic organisms are also examples of metabolic plasticity. In each case, the molecular mechanisms regulating these metabolic responses is poorly understood.

These inducible responses to changing environmental conditions are examples of homeostatic control mechanisms, which increase in importance during succession as direct organismal control over resources increases (Margalef, 1968; Odum, 1969). In the complex network design of aquatic communities, it is reasonable to suggest that many molecular "fine-tuning" mechanisms have evolved which regulate aspects of community growth dynamics. Examination of the molecular ecology of phytoplankton species and natural aquatic communities is an area of research which is just beginning to be explored. This study is an investigation into the occurrence and potential functions of cyclic adenosine 3':5'-monophosphate (cAMP), a potent and ubiquitous metabolic regulatory molecule in heterotrophic organisms, in phytoplankton and in natural aquatic communities.

Involvement of cAMP in Heterotrophic Organisms

Much information exists on the ubiquitous presence of cAMP in both eukaryotic and prokaryotic heterotrophs, and its versatility as a metabolic regulatory agent. In animals, cAMP is the intracellular messenger which regulates the activity of a variety of hormones (Robison, et al., 1971a) and non-endocrine functions (Greengard and Costa, 1970; Robison, et al., 1971b; Pastan, et al., 1975). The collective evidence suggests that cAMP functions in animals through the activation of cAMP-dependent protein kinases, which in turn activate or inactivate other specific enzyme systems (reviewed by Greengard and Robison, 1973).

Regulation of cAMP levels in biological systems is a function of both the rate of synthesis and the rate of degradation. In organisms examined to date, cAMP is produced from ATP through the action of adenyl cyclase, an enzyme which is generally membrane-bound (Robison, et al., 1971a). In cAMP-mediated systems, extracellular signals interact with the regulatory subunit of adenyl cyclase, resulting in increased production of cAMP. The resultant accumulation of cAMP intracellularly functions as a second messenger involved in homeostatic responses in the cell. Degradation of cAMP is largely a function of hydrolysis to 5'-AMP through the action of cyclic 3':5'-nucleotide phosphodiesterase, a soluble enzyme with a high degree of specificity for 3':5'-cyclic nucleotides (Robison, et al., 1971a). This enzyme may also be released extracellularly by cells as a mechanism for reducing extracellular cAMP concentrations (Konijn, et al., 1969). Direct extracellular release of cAMP also occurs in some organisms, but its function is poorly understood (Makman and Sutherland, 1965).

A generalized effect of cAMP in heterotrophic organisms is that it signals changes in the extracellular environment. In this manner, cAMP

functions as an intra- and extracellular signal of impending "hard times" in terms of the overall metabolic economy of organisms and the need for appropriate homeostatic responses (Wicks, 1974). Cyclic AMP appears to be involved only in the regulation of inducible as opposed to constitutive processes (Robison, et al., 1971a). Further, the response that cAMP elicits in a given organism is a function of the cell or tissue type involved and the stage of growth of the cells. Thus, extrapolation of cause-and effect relationships between different cell types or organisms is often difficult or impossible.

The role of cAMP in bacterial cells is in many ways better understood than in animals, primarily because of the power of mutational analysis as well as the comparative speed and ease in doing experiments with bacteria. Aspects of nitrogen, phosphorus, and carbon metabolism are regulated by cAMP in a wide variety of bacteria. In Azotobacter, nitrogenase production is not directly stimulated by cAMP, but when nitrogenase production is repressed by NH_3, cAMP accelerates the rate of derepression (Lepo and Wyss, 1974) by a mechanism which requires protein synthesis. In Klebsiella aerogenes, cAMP regulates histidine utilization via the induction of glutamine synthetase (Prival, et al., 1973). Similarly, several aspects of glutamine metabolism are activated in E. coli in vitro by cAMP (Prusiner and Stadtman, 1971).

Many phosphorylases, phosphorylase kinases, phosphatases, and phosphorylase phosphatases are regulated by endogenous or exogenous cAMP in animal cells (reviewed by Greengard and Robison, 1972, 1973, 1974, 1976). Adenyl cyclase activity is stimulated by potassium phosphate in some bacteria and is inhibited by pyrophosphate in E. coli (Tao and Lipmann, 1969).

The regulation of carbon metabolism by cAMP in bacteria involves the removal of catabolite repression of inducible enzymes which are repressed by glucose. The enzymes required to metabolize glucose in bacteria are constitutive; those required to metabolize other sugars and energy-yielding compounds are inducible. The latter include enzymes for the metabolism of lactose, galactose, tryptophan, and other substances. Only when glucose in the extracellular medium is exhausted are these inducible enzymes synthesized. Makman and Sutherland (1965) first demonstrated that glucose lowered the intracellular level of cAMP in E. coli. Subsequent work demonstrated that cAMP could overcome the repression by glucose of the synthesis of β-galactosidase, as well as a number of other inducible enzymes associated with the catabolism of exogenous carbon sources (Pastan and Perlman, 1968; DeCrombrugghe, et al., 1969; Pastan, et al., 1971). Through the work of a number of different investigators, it became evident that cAMP promotes activation of catabolite-repressible proteins by a mechanism which differs fundamentally from protein kinase activation mechanisms in higher organisms (Pastan and Perlman, 1968, 1969, 1970; Perlman and Pastan, 1968, 1971; Zubay, et al., 1970; Anderson, et al., 1971; Pastan, et al., 1971; Beckwith, et al., 1972; Gondo, et al., 1978). In microbial cells, cAMP forms a complex with a specific protein dimer present in the cytoplasm, termed "cAMP receptor protein", or "CRP". The cAMP•CRP complex then attaches to the promotor site of a catabolite-sensitive operon, facilitating transcription of structural genes by mRNA. In this manner, glucose regulates the transcriptional rate of mRNA specific to the inducible enzymes by controlling the intracellular levels of cAMP. To date, there is no solid evidence that bacteria contain cAMP-dependent protein kinases or that eukaryotes contain cAMP receptor proteins which function at the transcriptional level of protein synthesis.

Although cAMP exerts its primary effects in cells as a second messenger, cAMP may also function in some cell types as a primary intra- or extracellular messenger. The permeability of cell membranes to certain metabolites, including uracil, calcium, magnesium, and potassium, is mediated by cAMP in some prokaryotic and eukaryotic cells (Judewicz, et al., 1974; Greengard and Robison, 1972). In Dictyostelium discoidium, a cellular slime mold, extracellular cAMP gradients, resulting from active release of cAMP by cells and breakdown of cAMP by externally-released cyclic 3':5'-nucleotide phosphodiesterase, function as chemotactic signals which result in aggregation of the cells in unfavorable environmental conditions (Konijn, et al., 1967). The release of cAMP by E. coli functions as a chemical attractant to cellular slime molds which feed on them (Konijn, 1969).

Involvement of cAMP in Photosynthetic Organisms

In contrast to the large body of data concerning cAMP in heterotrophic organisms, the occurrence and function of cAMP in photosynthetic organisms are poorly understood. To the casual reader of the literature, it would appear that cAMP has unequivocably been shown to occur in higher plants and that it has numerous functions in these organisms. However, the vast majority of investigations purporting to demonstrate the existence of cAMP in photosynthetic organisms are rendered unconvincing by basic flaws in methodology and interpretation of results.

Tomkins (1975) presented a general hypothesis on the origin of intercellular communication based on the presumed function of cAMP as an intracellular "symbol" of the state of the carbon source in the cells environment. Although Tomkins did not directly consider plants, many

botanists readily agreed that for "evolutionary reasons", cAMP must exist in plants and have metabolic significance. In heterotrophic organisms, cAMP controls the direction and pathways of metabolism according to the availability of external organic carbon sources. However, photosynthetic organisms, which require no external organic carbon source for energy transduction, have no need for this form of control. Thus, if cAMP occurs in plants, it may serve functions different from those observed in heterotrophs. However, cells within a plant can be heterotrophic with respect to other CO_2-fixing cells in the same plant, e.g. roots, young leaves. Thus, in certain plant cells, cAMP may have functions similar to those known in heterotrophic organisms.

Labeling techniques using ^{14}C-adenine (Pollard, 1970), the Gilman protein binding assay (Gilman, 1972), and the protein kinase stimulation assay (Kuo and Greengard, 1972) have been used to assay plant materials for cAMP (reviewed by Lin, 1974; Amrhein, 1977). These techniques, although suitable for the assay of cAMP from heterotrophic organisms, produce positive but erroneous results in plant extracts in the absence of rigorous purification techniques (Bressan, et al., 1976). Despite several attempts to isolate it, adenyl cyclase activity has not yet been convincingly demonstrated in any tissue of higher plants (Robison, et al., 1971a; Lin and Varner, 1972). Further, cyclic 3':5'-nucleotide phosphodiesterase which has been isolated from plant tissues differs in many biochemical respects from diesterase isolated from bacterial and mammalian cells, most notably in its lack of specificity for cyclic 3':5'-nucleotides (reviewed by Lin, 1974; Amrhein, 1977).

Strong evidence has been presented for the occurrence of cAMP in sterile cultures of the angiosperm Lolium multiflorum (ryegrass) (Ashton and Polya, 1978), the moss Funaria hygrometrica (Handa and Johri, 1977),

the green alga Chlamydomonas reinhardtii (Amrhein and Filner, 1973; Bressan, et al., 1980b), the blue-green alga Anabaena variabilis (Hood, et al., 1979), and the chrysophycean alga Ochromonas malhamensis (Bressan, et al., 1980a). In the latter three organisms, considerable quantities of cAMP were found in the extracellular media. These investigations demonstrated that the Gilman protein binding assay and the protein kinase luciferin-luciferase assay (Handa and Bressan, 1980) could be used to assay cAMP in plant materials which had been purified with neutral alumina and Dowex 50 chromatography.

Little is known of the role of cAMP in photosynthetic organisms. In view of the methodological limitations which plagued early studies on cAMP in higher plants, no conclusions can be drawn from the numerous reports which purport to demonstrate metabolic effects of cAMP in higher plants. Several investigators, for instance, have proposed that cAMP may "mimic" the action of a wide variety of plant hormones (Salomon and Mascarenhas, 1971; Hall and Galsky, 1973; Rast, et al., 1973; Truelsen, et al., 1974), but these results are far from convincing. Amrhein and Filner (1973) demonstrated the presence of a theophylline-sensitive cyclic 3':5'-nucleotide phosphodiesterase in Chlamydomonas reinhardtii, and provided strong evidence that increased intracellular cAMP concentrations were involved in flagellar function and regeneration in this alga. Hood, et al. (1979) found that nitrogen starvation in Anabaena variabilis resulted in increased levels of intra- and extracellular cAMP, immediately preceding heterocyst formation. Although Borowitzka and Volcani (1977) reported that cAMP is involved in silica metabolism in Cylindrotheca fusiformis, and Berchtold and Bachofen (1977) reported that cAMP regulates chlorophyll synthesis in Chlorella fusca, both papers reported inconclusive results.

Evidence for the Occurrence of cAMP in Natural Aquatic Systems

The existence of cAMP in natural ecosystems, either aquatic or terrestrial, has not been investigated before the study reported in this thesis. In view of the evidence that at least some phytoplankton species produce cAMP and release it extracellularly in culture, it was reasonable to suspect that similar processes might occur in nature. Organic compounds, including organic phosphates, are actively released from healthy algal cells and may account for a substantial proportion of the labile dissolved organic carbon present in lakewater (Fogg, 1962; Hellbust, 1965; Aaronson, 1971). It has been postulated that these excreted molecules may serve as nutrient sources or as regulatory molecules important in various life cycle phenomena.

Lean (1973) reported that phytoplankton populations released a low molecular weight (ca. 250 daltons) phosphate ester into lakewater during periods of high phytoplankton population density. Francko and Heath (1979) demonstrated that much of the dissolved phosphorus present in eutrophic lakewater existed as low molecular weight phosphate esters, and that the composition of the phosphate ester pool varied dynamically during the year.

Collectively, the evidence suggests that cAMP may exist in aquatic systems, both associated with plankton and dissolved in lakewater. In view of the myriad functions that cAMP regulates in organisms examined to date, the occurrence of cAMP in aquatic systems could have profound physiological and ecological consequences at the organismal, population, community, and ecosystems levels of organization.

Objectives of the Investigation

The research in this investigation was designed to test the thesis that cAMP production and extracellular release is widespread among phytoplanktonic algae, that naturally-occurring phytoplankton in lake systems produce and release cAMP, and that the production and release of cAMP by these organisms has physiological and ecological significance. The range of potential effects in which cAMP could reasonably be involved in phytoplanktonic algae is very large, rendering intensive investigations into any single phenomenon inefficient. With this limitation in mind, I have designed my research to address the following questions:

1) Do phytoplankton species common to temperate lake systems produce cAMP and release it extracellularly? If so, are there interspecific differences in production and release under similar environmental conditions, or intraspecific differences in production and release during different stages of growth?

2) Is the production and release of cAMP by cultured phytoplankton related to the availability of inorganic nitrogen or phosphorus? If so, is differential cAMP production or release related to the induction of alkaline phosphatase activity or to heterocyst formation?

3) Is cAMP involved in the regulation of primary productivity, chlorophyll synthesis, or growth rate dynamics in cultured phytoplankton?

4) Does cAMP exist in natural aquatic systems? If so, can the seasonally dynamic production and extracellular release of cAMP by the epilimnetic planktonic community be related to differences in trophic dynamic structure between oligotrophic and eutrophic lake systems?

5) Can the seasonal dynamics of particulate-associated or dissolved cAMP

in oligotrophic or eutrophic lake systems be related to changes in phytoplanktonic community structure, primary productivity, alkaline phosphatase activity, or chlorophyll synthesis during the year in these systems? 6) Do the levels of cAMP differ within the pelagial and littoral zones of lakes, both on a seasonal basis and between lakes of differing trophic status?

To address these questions, this investigation was divided into laboratory and *in situ* field experiments. Laboratory-cultured phytoplankton, characterized fully in Chapters I and II, were grown under both optimal and suboptimal nutrient regimes under constant temperature and illumination regimes. Cellular and extracellular cAMP production, characterized by a number of biochemical techniques, was correlated with growth rate dynamics, chlorophyll *a* synthesis, ^{14}C-bicarbonate uptake, alkaline phosphatase activity, and heterocyst formation. The blue-green alga *Anabaena flos-aquae* was used as a model system in the examination of these metabolic variables. Additionally, this alga was used to test the effects of perturbation of cAMP levels on the aforementioned metabolic variables.

Investigations on the occurrence and seasonal dynamics of cAMP in aquatic systems were conducted on Lawrence Lake, a hardwater oligotrophic lake, and on Wintergreen Lake, a hardwater hypereutrophic lake, both in southwestern Michigan. These systems were chosen because of their differences in trophic status and because of the large body of research (Wetzel, 1975) relating to these systems. Putative cAMP from both systems was characterized by several biochemical techniques. Weekly sampling of particulate and dissolved cAMP in the epilimnia of both lakes was correlated with data on the rates of primary productivity, alkaline phosphatase activity, chlorophyll *a* synthesis and changes in phytoplankton community structure.

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CHAPTER I

CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE: ISOLATION AND CHARACTERIZATION FROM GREEN AND BLUE-GREEN ALGAE

INTRODUCTION

The occurrence of cyclic adenosine 3':5'-monophosphate (cAMP) in photosynthetic organisms remains controversial (Lin, 1974; Amrhein, 1977). Strong evidence has been presented for the occurrence of low concentrations of cAMP in sterile cultures of the angiosperm Lolium multiflorum (ryegrass; Ashton and Polya, 1978), the moss Funaria hygrometrica (Handa and Johri, 1977), the green alga Chlamydomonas reinhardtii (Amrhein and Filner, 1973; Bressan, et al., 1980b), the chrysophycean alga Ochromonas malhamensis (Handa and Bressan, 1978; Bressan, et al., 1980a), and the blue-green alga Anabaena variabilis (Hood, et al., 1979). In the latter three organisms, cAMP was found in large quantities in the extracellular media. Firm evidence that phytoplankton produce and release cAMP is a necessary prelude to understanding the potential role of cAMP in phytoplanktonic metabolism.

This investigation reports evidence, on the basis of several criteria, that cAMP production and release may be widespread among planktonic algae common to temperate lake systems.

MATERIALS AND METHODS

Strains of the blue-green algae Microcystis aeruginosa Kutz. (UTEX 1937) and Synechococcus leopoliensis (Raeib.) Komarek (UTEX 625) and the green algae Chlorella pyrenoidosa Chick (UTEX 343), Cosmarium botrytis Meneg. (UTEX 75), Pandorina morum Bory (UTEX 18), and Pediastrum

biradiatum Meyen (UTEX 37) were obtained from the University of Texas culture collection (Starr, 1978). A strain of Anabaena flos-aquae (Lyng.) Breb. isolated from Lake Erie, was obtained from Dr. G.-Y. Rhee, New York State Dept. of Health, and a strain of Scenedesmus communis Hegewald (=quadricauda) was isolated from Gull Lake in southwestern Michigan. Each strain was rendered axenic where necessary, cloned, and maintained on Moss agar slants (Moss, 1972). Aliquots (25 ml) of stationary phase stock cultures of each clone were inoculated into 2500-ml sterile Moss medium (Moss, 1972), and grown to late exponential phase.

Illumination was provided by cool white fluorescent light (Luxor Vita Lite) at an intensity of 43 $\mu E\ m^{-2}\ s^{-1}$ on a 12 h on, 12 h off cycle, in environmental chambers maintained at 20°C. Culture flasks (4000 ml) were placed randomly in each chamber and cells were grown without shaking. Cell densities at the late exponential phase ranged from 3×10^5 cells ml^{-1} (Pandorina) to 8×10^6 cells ml^{-1} (Synechococcus).

Cultured cells (2500 ml) were harvested on a Sorvall model SS-3 continuous-flow centrifuge at 10,000 g using a flow-through rate of 180 ml min^{-1}. Filtration of effluent water and microscopic examination of pellet aliquots and effluent water demonstrated that this sedimentation force quantitatively removed phytoplankton without rupturing cells.

Culture filtrate samples were collected by gently filtering 100-ml aliquots of each culture through pre-washed 0.5 μm pore size Reeve-Angel glass-fiber filters using a vacuum differential of 0.5 atm. Cultures were verified as axenic before harvest by inoculating aliquots onto nutrient and plate count agars and by examination by phase contrast

microscopy.

Centrifuged algal cells were extracted in 0.5 M $HClO_4$ (10 ml g^{-1} cells) at 0°C, purified (Handa and Bressan, 1978, 1980; Bressan, et al., 1980a,b), and redissolved in 50 mM Tris-HCl, pH 6.8. Cyclic AMP present in culture filtrate samples was recovered by adsorption and elution from purified (Handa and Johri, 1977) Norit A and were redissolved in 50 mM Tris-HCl, pH 6.8 (Handa and Bressan, 1978, 1980; Bressan, et al., 1980a,b). All samples in 50 mM Tris-HCl were further purified by neutral alumina and Dowex 50 chromatography, lyophilized, and redissolved in distilled, deionized water (Handa and Bressan, 1978, 1980; Bressan, et al., 1980a,b), and assayed for cAMP. Radioactivity was measured in Insta-Gel (Packard Co.) scintillation fluid on a Beckman model 8000 liquid scintillation counter. Recoveries of ^{3}H-cAMP internal standard in both cell and filtrate samples ranged from 10-30%.

Cyclic AMP binding protein, isolated from bovine heart (Kuo and Greengard, 1972) was used to assay cAMP by the Gilman protein binding assay (Gilman, 1972). Samples were boiled for 3 min. before assaying to denature interfering substances present even after extensive purification (R. E. Bressan, personal communication). Contamination by cAMP present in glassware and reagents never exceeded the detectability limits of the assay (0.01 pmole assay $tube^{-1}$). Cyclic AMP-dependent protein kinase from bovine heart was used to measure cAMP by the luciferin-luciferase method of Handa and Bressan (1980). The amount of ATP remaining in each reaction mixture was measured by the firefly luciferin-luciferase system. Luminescence was measured with an ATP photometer (Aminco 4-7441) coupled to a digital peak integrator (Columbia Scientific Industries 208).

The kinetics of cyclic 3':5'-nucleotide phosphodiesterase inactivation of cAMP measured by the Gilman assay were determined according to Handa and Johri (1977) and Bressan, et al., (1980a,b). Lyophilized Dowex 50 samples were chromatographed by silica gel thin-layer chromatography. The resulting cAMP fraction (R_f = 0.75) was digested with phosphodiesterase. Controls containing theophylline (1,3-dimethyl xanthine), a specific inhibitor of cyclic 3':5'-nucleotide phosphodiesterase, were measured concurrently.

RESULTS AND DISCUSSION

Each alga produced and released into the medium a factor which co-purified with authentic cAMP on neutral alumina, Dowex 50, and silica gel chromatography, replaced authentic cAMP in the Gilman and protein kinase assays, and which was degraded by cyclic 3':5'-nucleotide phosphodiesterase at the same rate as authentic cAMP. Cellular and extracellular cAMP levels were measured equally well by either the protein kinase assay or the Gilman assay (Table 1). Considerable variation in cAMP levels in different algae was noted. Cellular cAMP concentrations ranged from 90.3 $\pm$ 5 pmoles g^{-1} fresh wt in Synechococcus to 394.3 $\pm$ 18 pmoles g^{-1} fresh wt in Pandorina (Gilman assay). Similar variability was noted in extracellular cAMP levels (Table 1), which ranged from 8.8 $\pm$ 2 pmoles $liter^{-1}$ in Synechococcus to 207 $\pm$ 10 pmoles $liter^{-1}$ in Pediastrum. These differences were not due to random sampling error. The coefficient of variation in cultures of Anabaena (Gilman assay) (N=4) was found to be 8.9% and 7.4% for cellular and extracellular cAMP levels, respectively. Care was taken to harvest cultures at the same stage in growth in each case, but slight

Table 1. Cyclic AMP concentrations occurring in freshwater planktonic algae and released extracellularly into the media as determined in purified extracts by the protein kinase assay (PKL) and the Gilman assay. Cell values are in pmoles g^{-1} fresh weight ± 1 SD (n=4); filtrate values in pmoles $liter^{-1}$ ± 1 SD (n=4). Values in parentheses equal to % difference in predicted Gilman assay value when 0.5 pmoles cAMP was added to each assay tube as an internal standard. The ratio between media cAMP in pmoles l^{-1} and cellular cAMP in pmoles l^{-1} was determined (Gilman assay values).

Source	PKL Assay		Gilman Assay		
	Cells	Media	Cells	Media	Media / Cellular ($cAMP l^{-1}$)
Blue-green algae:					
Anabaena flos-aquae	90 ± 18	480 ± 96	92 ± 5 (+ 2%)	440 ± 20 (+ 8%)	11.0
Synechococcus leopoliensis	90 ± 18	9 ± 4	90 ± 5 (- 1%)	9 ± 2 (+ 3%)	0.30
Microcystis aeruginosa	338 ± 67	43 ± 8	348 ± 17 (+10%)	52 ± 3 (+10%)	0.60
Green algae:					
Scenedesmus communis	160 ± 32	120 ± 24	155 ± 8 (+ 5%)	150 ± 8 (+ 3%)	2.2
Chlorella pyrenoidosa	102 ± 20	45 ± 9	108 ± 6 (- 2%)	36 ± 4 (+ 5%)	1.1
Cosmarium botrytis	135 ± 27	58 ± 12	117 ± 6 (+10%)	56 ± 3 (- 2%)	2.4
Pandorina morum	386 ± 79	179 ± 36	394 ± 18 (+ 9%)	202 ± 10 (+10%)	2.0
Pediastrum biradiatum	132 ± 26	183 ± 36	150 ± 8 (+ 0%)	207 ± 10 (+10%)	4.6

differences in synchrony between cultures may have accounted for some variability. The evidence indicated that significant species-to-species differences occurred in cAMP production and release under similar growth conditions. In most algal cultures, the majority of cAMP was found dissolved in the culture media (Table 1).

Aliquots of purified cellular and media samples were augmented with authentic cAMP (0.5 pmole assay tube^{-1} in samples containing from 0.1-3.2 pmole assay tube^{-1}) before analysis by the Gilman assay, to demonstrate that non-specific interference did not occur. Additive cAMP levels in both cellular and extracellular samples never varied by more than 10% from predicted values, further evidence that the Gilman assay measured authentic cAMP (Table 1).

When purified cellular samples from Anabaena and Scenedesmus were incubated with cyclic 3':5'-nucleotide phosphodiesterase, protein binding activity was abolished at the same rate as with authentic cAMP (Figure 1). Furthermore, theophylline completely inhibited diesterase hydrolysis of both authentic cAMP and putative cAMP from the same organisms (Figure 1).

CONCLUSIONS

The collective evidence indicates that cAMP production may be widespread in phytoplankton and that significant release of cAMP may occur. The extracellular levels of cAMP noted may have resulted from active cellular release or cell lysis, but little detritus was noted in microscopic analysis of culture samples, indicating that lysis in the actively growing cultures was probably minimal. The production and extracellular release of cAMP varied greatly among different

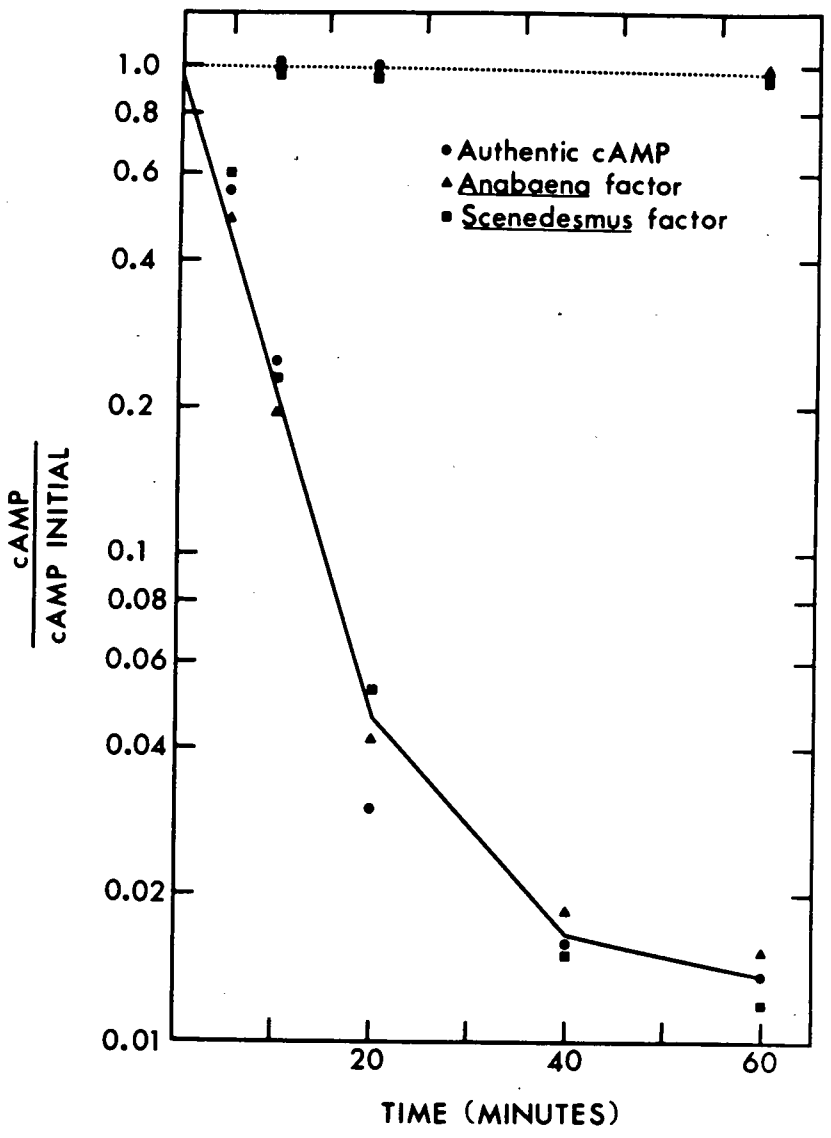

Figure 1. Kinetics of cyclic 3':5'-nucleotide phosphodiesterase-catalysed hydrolysis of authentic cAMP and putative cAMP isolated from Anabaena and Scenedesmus cells (——————). Shown is the fraction of original cAMP remaining in the reaction mixture as a function of incubation time. Hydrolysis kinetics in the presence of 5 mM theophylline (••••••••••••••). All values were determined by the Gilman assay.

phytoplankton species grown under the same environmental conditions and harvested at the same stage in growth. Further work on the dynamics of cAMP in phytoplankton and its potential role in phytoplanktonic metabolism is clearly warranted.

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CHAPTER II

EVIDENCE FOR THE INVOLVEMENT OF cAMP IN THE METABOLISM OF *ANABAENA FLOS-AQUAE*

INTRODUCTION

Although cyclic adenosine 3':5'-monophosphate (cAMP) has been isolated from phytoplankton (Amrheim and Filner, 1973; Hood, *et al.*, 1979; Bressan, *et al.*, 1980a,b; Francko and Wetzel, 1980; Chapter I of this dissertation) little is known of the physiological significance of cAMP in these organisms. Amrheim and Filner (1973) postulated that increased intracellular cAMP concentrations in *Chlamydomonas reinhardtii* may be involved in flagellar regeneration and function. Hood, *et al.*, (1979) demonstrated that nitrogen starvation of *Anabaena variabilis* resulted in an increase in both cellular and extracellular cAMP, immediately preceding heterocyst formation.

This investigation presents evidence that the concentrations of both intra- and extracellular cAMP vary markedly during the life cycle of the blue-green alga *Anabaena flos-aquae* and that cAMP may be involved in the dynamics of ^{14}C-bicarbonate uptake, heterocyst formation, phosphate limitation and growth rate at certain stages of growth.

MATERIALS AND METHODS

General Culture Design

A strain of the blue-green alga *Anabaena flos-aquae* (Lyng) Breb, isolated from Lake Erie, was obtained from Dr. G.-Y. Rhee, New York State Dept. of Health. An axenic clone of this strain was isolated and maintained on Moss agar slants (Moss, 1972).

All culture experiments were conducted in environmental chambers

maintained at 20°C. Illumination was provided by cool white fluorescent light (Luxor Vita Lite) at an intensity of 43 $\mu E\ m^{-2}\ s^{-1}$ on a 12 h on, 12 h off cycle. Cultures were agitated gently on reciprocal shakers in all experiments. Each culture was verified as axenic prior to harvest by inoculating aliquots of each culture on nutrient and plate count agars and by examination under phase contrast microscopy.

Modification of Culture Conditions Utilized

Standard Moss media (Moss, 1972) was used to maintain stock cultures of the cloned cell line used in these experiments. The first series of experiments were designed to assess the culture-to-culture variability of intra- and extracellular cAMP in cultures of Anabaena grown under optimal nutrient conditions and harvested at the same stage of growth. Accordingly, aliquots of stock cultures (25 ml) were inoculated into four culture flasks (250 ml) containing standard Moss media, grown to stationary phase, and harvested.

In the second series of experiments, the effects of differential N:P ratios on the production and release of cAMP and other physiological parameters in Anabaena was tested. In these experiments the following modification of Moss media, normally containing 30 $\mu M\ P\ l^{-1}$ as PO_4-P, and 360 $\mu M\ N\ l^{-1}$ as NO_3-N, were employed:

1) N:P = 80:1 established by preparing Moss media with 3 $\mu M\ P\ l^{-1}$ and 240 $\mu M\ N\ l^{-1}$;

2) N:P = 10:1 established by preparing media with 3 $\mu M\ P\ l^{-1}$ and 30$\mu M\ N\ l^{-1}$; and

3) N:P = 2:1 by preparing media with 3 $\mu M\ P\ l^{-1}$ and 6 $\mu M\ N\ l^{-1}$.

Each N:P ratio was prepared in duplicate cultures (2500 ml), inoculated with aliquots (25 ml) of stationary phase stock culture, grown to

stationary phase and harvested.

The third series of experiments was designed to assess the effect of nitrogen and orthophosphate limitation on the short-term production and release of cAMP by actively growing cultures of Anabaena. Aliquots (25 ml) of stationary phase stock cultures were inoculated into 2500 ml sterile Moss media, grown to late exponential phase, and harvested by continuous-flow centrifugation (Sorvall model SS-3). Centrifugation resulted in eight pellet fractions which were processed by one of the following procedures. Two fractions were immediately acidified, purified and assayed for cellular cAMP. The remaining six fractions were resuspended in 125 ml sterile Moss media containing one of the following modifications: A) no inorganic phosphate; B) no inorganic nitrate; C) no inorganic nitrate or phosphate; D) standard Moss media. At 1, 8, and 24 h post-inoculation, two cultures were randomly selected, harvested and assayed for chlorophyll a, ^{14}C-bicarbonate uptake, alkaline phosphatase activity, heterocyst number, cell density, total biomass, and cellular and extracellular cAMP.

The fourth series of experiments was designed to assess the effects of perturbations of cAMP metabolism on the production and extracellular release of cAMP by actively growing Anabaena and the effect of resultant differential cAMP production and release on the aforementioned physiological variables.

Accordingly, several modifications of standard Moss media utilizing inhibitors of cAMP metabolism were used. Methylxanthine compounds are potent inhibitors of cyclic 3':5'-nucleotide phosphodiesterase in heterotrophic organisms (Robison, et al., 1971) and in at least one eukaryotic algal species examined to date (Amrheim and Filner, 1973).

In this experiment, MIX (1-methyl 3-isobutyl xanthine; Sigma Chemical Co.) was used in an attempt to inhibit cyclic 3':5'-nucleotide phosphodiesterase activity in cultures of Anabaena. Accumulation of both intracellular and extracellular cAMP should then result, although the presence of a methylxanthine-sensitive cyclic 3':5'-nucleotide phosphodiesterase has not previously been demonstrated in any prokaryotic organism. Crystalline cyclic 3':5'-nucleotide phosphodiesterase (Sigma Chemical Co.) was utilized to reduce the concentration of extracellular cAMP released by Anabaena. Alternately, crystalline cAMP (Sigma Chemical Co.) was added to culture media, to augment cAMP released by Anabaena cells.

In cAMP-perturbation experiments, cells were grown to late exponential phase in 2500 ml sterile Moss media and harvested as previously described. The resultant eight pelleted fractions were then processed in the following manner. Two fractions were immediately acidified, purified and assayed for cAMP. Duplicate fractions were then inoculated into sterile Moss media (100 ml) containing the following additions: 1) 5 mM MIX: 2) 0.1 unit cyclic 3':5'-nucleotide phosphodiesterase; 3) 1600 pmoles l^{-1} cAMP; 4) standard Moss media.

Harvesting of Cell Cultures

Cell cultures were harvested by one of two methods: 1) Conventional centrifugation at 10,000 g for 10 min in a Sorvall model SS-3 centrifuge, which was used in 100-250-ml cultures, or 2) Continuous-flow centrifugation at 10,000 g using a flow-through rate of 180 ml min^{-1}. Filtration of effluent water and supernatants, and microscopic examination of pellet aliquots and effluent water demonstrated that this sedimentation force quantitatively (> 95%)

removed phytoplankton without rupturing cells.

To preclude the need for determining fresh weight biomass from pelleted cells after each culture was harvested, a standard curve was prepared which related culture absorbance (A_{600} nm) to pelletable biomass. This relationship was linear within a range from 0.05-0.4 g tissue 100-ml^{-1} culture. This modification was used because of the necessity of immediate acid fixation of cell samples following harvest.

Measurement of Intracellular and Extracellular cAMP

Culture filtrate samples and centrifuged algal cells were purified and assayed for cAMP essentially according to the methods of Bressan, et al. (1980a,b), Handa and Bressan (1980), Francko and Wetzel (1980) and Chapter I of this dissertation. Culture filtrate samples were collected by gently filtering 100-ml aliquots of supernatant or flow-through water from each culture through pre-washed 0.5-µm pore sized Reeve-Angel glass fiber filters. Cyclic AMP present in culture filtrate samples was recovered by absorption and elution from purified Norit A and were redissolved in 50 mM Tris-HCl, pH 6.8. Centrifuged algal cells were extracted in 0.5 N $HClO_4$ (10 ml g^{-1} cells) at 0°C, purified, and redissolved in 50 mM Tris-HCl, pH 6.8. All samples in 50 mM Tris-HCl were further purified by neutral alumina and Dowex 50 chromatography, lyophilized, redissolved in distilled, deionized water and assayed for cAMP by the Gilman protein binding assay (Gilman, 1972). This assay has been shown effective in the assay of cAMP from algal samples provided that each sample is purified with neutral alumina and Dowex 50 chromatography prior to assay (Bressan, et al., 1980a,b; Francko and Wetzel, 1980; Chapter I of this dissertation).

Radioactivity was measured in Insta-gel (Packard Co.) scintillation

fluid on a Beckman model 8000 liquid scintillation counter. Recoveries of ^{3}H-cAMP internal standard in both cell and filtrate samples varied from 3-20%.

Determination of Chlorophyll a Content, ^{14}C-bicarbonate Uptake, Alkaline Phosphatase Activity, and Culture Turbidity

Chlorophyll a content was determined on aliquots (5 to 20 ml) of each culture prior to harvest. Aliquots were filtered through 0.8-μm pore size Millipore (AA) filters and the chlorophyll a content of each filter, corrected for phaeophytin, was assayed on a Hitachi-Perkin-Elmer model 139 spectrophotometer by the trichromatic assay of Wetzel and Likens, 1979. Chlorophyll concentrations were then expressed as μg chlorophyll a g^{-1} fresh weight cells. ^{14}C-bicarbonate uptake rates were measured by the method of Wetzel and Likens (1979) by inoculating 200 μl aliquots of each culture prior to harvest in 10-ml Moss media (pH 7.8) containing 100 μl ^{14}C-bicarbonate (sp. act=100 μCi ml^{-1}) for 1 h under the environmental conditions previously described. Inoculated samples were then acidified with 100 μl of concentrated sulfuric acid and allowed to equilibrate for 15 min. Each sample was then lyophilized, redissolved in 1 ml distilled, deionized water, and CPM of incorporated ^{14}C were converted to units of DPM X 10^{-8} g^{-1} fresh wt h^{-1}.

Alkaline phosphatase activity was measured by the fluorometric method of Kuenzler and Parras (1965) on 0.5-ml aliquots of each cell culture prior to harvest. Fluorescence was measured on a Turner model 111 fluorometer and converted to units of μmoles P released X 10^{-4} g^{-1} fresh wt min^{-1}.

Culture absorbance was measured at 600 nm using a Spectronic 20

spectrophotometer prior to harvest of each cell culture. Measurements were reported as the mean of three determinations.

RESULTS AND DISCUSSION

Analyses of Stationary Phase Anabaena Cultures Under a Variety of Nutrient Conditions

An analysis of the variability of cellular and extracellular cAMP levels in stationary phase cultures of Anabaena grown under optimal nutrient conditions revealed a coefficient of variation of 8.9% and 7.4% for cellular and extracellular cAMP (N=4), respectively (Table 1). Thus, cultures grown under the same conditions and harvested at the same stage in growth contained similar amounts of cellular and extracellular cAMP.

When cells grown to stationary phase in media containing differing initial N:P ratios were analyzed, significant differences in standing crop biomass, ^{14}C-bicarbonate uptake, alkaline phosphatase specific activity, cellular and extracellular cAMP and the ratio between extracellular and cellular cAMP l^{-1} were found (Table 2). Cells grown in media containing an N:P ratio of 80:1 (N=2) exhibited the highest alkaline phosphatase specific activity, ^{14}C-bicarbonate uptake rate, cellular and extracellular cAMP concentrations and the highest ratio of extracellular to cellular cAMP (Table 2). Cells grown in media with an N:P ratio of 10:1 (N=2) exhibited the lowest ^{14}C-bicarbonate uptake rate, an intermediate alkaline phosphatase activity and the lowest levels of cellular cAMP, extracellular cAMP and ratio of extracellular to cellular cAMP l^{-1} (Table 2). Cells grown in media containing an N:P ratio of 2:1 (N=2) exhibited the largest standing crop biomass, the lowest alkaline phosphatase specific activity, intermediate levels of

Table 1. Variation in cellular and extracellular cAMP concentration in stationary phase Anabaena flos-aquae, as determined by the Gilman assay. Values are in pmoles g^{-1} fresh wt (cellular) and pmoles $liter^{-1}$ (extracellular)

Culture No.	Cellular cAMP	Extracellular cAMP
1	29.3	1566
2	31.0	1764
3	26.0	1480
4	29.9	1602
	$\underline{x}$ = 29.1	$\underline{x}$ = 1603
	S.D. = 2.6	S.D. = 119
	V = 8.9%	V = 7.4%

Table 2. Effect of initial N:P ratios on cAMP and metabolic variables in stationary phase Anabaena. Biomass (g l^{-1}), chlorophyll a ($\mu g\ g^{-1}$), ^{14}C-bicarbonate uptake (DPM x $10^{-8} g^{-1} h^{-1}$ ± 1 S.D.; N=4), alkaline phosphatase specific activity (μmoles x $10^{4} g^{-1} min^{-1}$) were measured in duplicate cultures at each N:P ratio along with cellular cAMP (pmoles g^{-1} fresh wt ± 1 S.D.; N=2) and extracellular cAMP (pmoles l^{-1} ± 1 S.D.; N=2). The ratio of extracellular to cellular cAMP in each culture, a measure of relative cAMP release per cell, is also reported.

N:P Ratio	Culture No.	Biomass	Chlor a	^{14}C Uptake	Alkaline Phosphatase Activity	Cellular cAMP	Extracellular cAMP	Extracellular / Cellular cAMP l^{-1}
80:1	1	.32	320	9.9 ± 1.0	40	183 ± 15	435 ± 40	7.4
	2	.40	380	8.5 ± .6	38	138 ± 10	355 ± 35	6.5
10:1	1	.40	360	4.8 ± .8	33	122 ± 6	120 ± 6	2.5
	2	.40	360	5.6 ± .4	33	130 ± 7	125 ± 6	2.4
2:1	1	.48	400	6.8 ± 1.0	27	160 ± 8	310 ± 15	4.0
	2	.42	360	4.6 ± .4	24	142 ± 10	310 ± 15	4.6

cellular cAMP and extracellular cAMP, and an intermediate ratio of extracellular to cellular cAMP l^{-1} (Table 2).

Data on stationary phase Anabaena cultures grown in normal Moss media and the aforementioned N:P ratio variants were pooled (N=9) and correlation analyses were performed as the relationships between cellular and extracellular cAMP and alkaline phosphatase activity, chlorophyll a content, and ^{14}C-bicarbonate uptake. In stationary phase Anabaena cells, cellular cAMP levels were significantly correlated (r^2 = 0.80; $P < 0.05$) to ^{14}C-bicarbonate uptake rates by the exponential relationship ($y = ae^{bx}$) shown in Figure 1. Cellular cAMP could not be significantly correlated ($P >> 0.05$) to chlorophyll a content or alkaline phosphatase activity. Extracellular cAMP could not be significantly correlated ($P >> 0.05$) to either alkaline phosphatase activity, chlorophyll a content or ^{14}C-bicarbonate uptake rates. Growth rates (Figure 2a) as measured by absorbance could not be correlated to cellular or extracellular cAMP.

The combined data on stationary phase Anabaena cultures suggested that both extremely low and extremely high N:P ratios resulted in increased production and release of cAMP relative to production and release under balanced (N:P = 10:1) nutrient conditions. The range of cellular (29-185 pmoles g^{-1} fresh wt) and extracellular (120-1730 pmoles l^{-1}) was highly variable in cells collected at the same stage in growth, but grown under different N:P regimes, suggesting that differential nutrient availability may have affected cAMP production and release. The observation that ^{14}C-bicarbonate uptake was correlated to cellular cAMP by an exponential relationship under a wide range of N:P ratios suggested that cellular cAMP may have been involved in regulating some phase of differential C-fixation under differing nutrient stress.

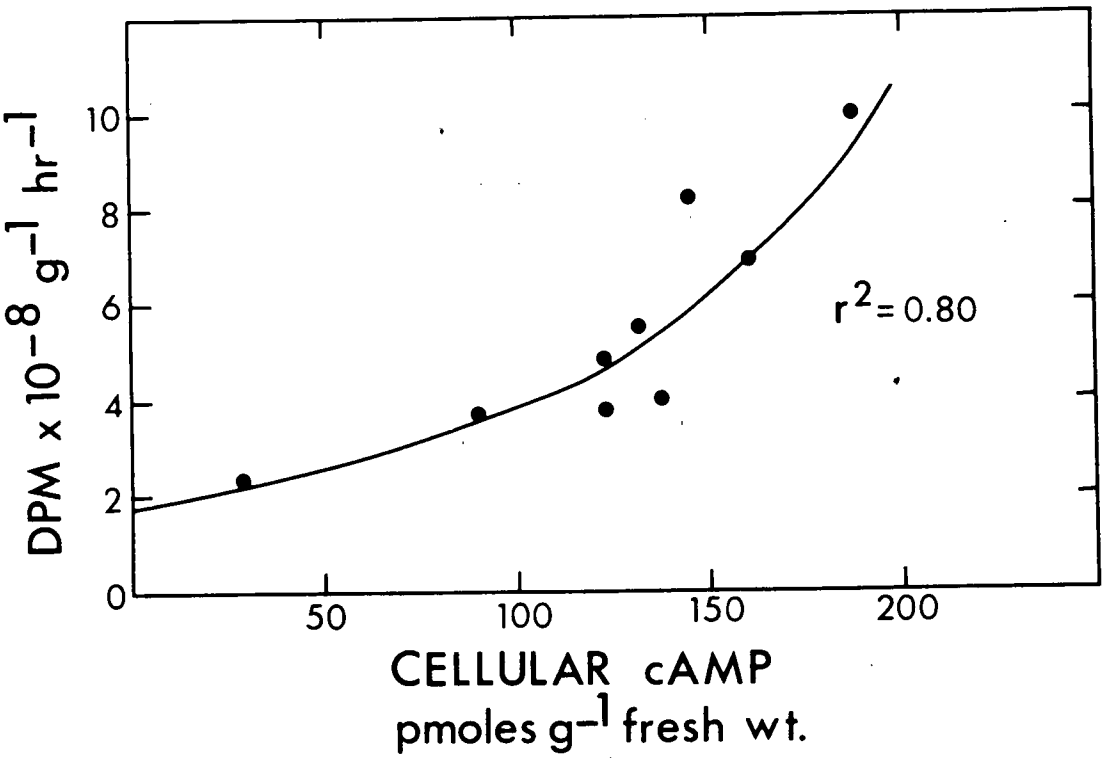

Figure 1. Exponential relationship ($y = ae^{bx}$) between cellular cAMP levels (pmoles g^{-1} fresh wt) and ^{14}C-bicarbonate uptake rates (DPM $\times 10^{-8} g^{-1}$ fresh wt h^{-1}) in stationary phase cultures of Anabaena flos-aquae under differing N:P ratios.

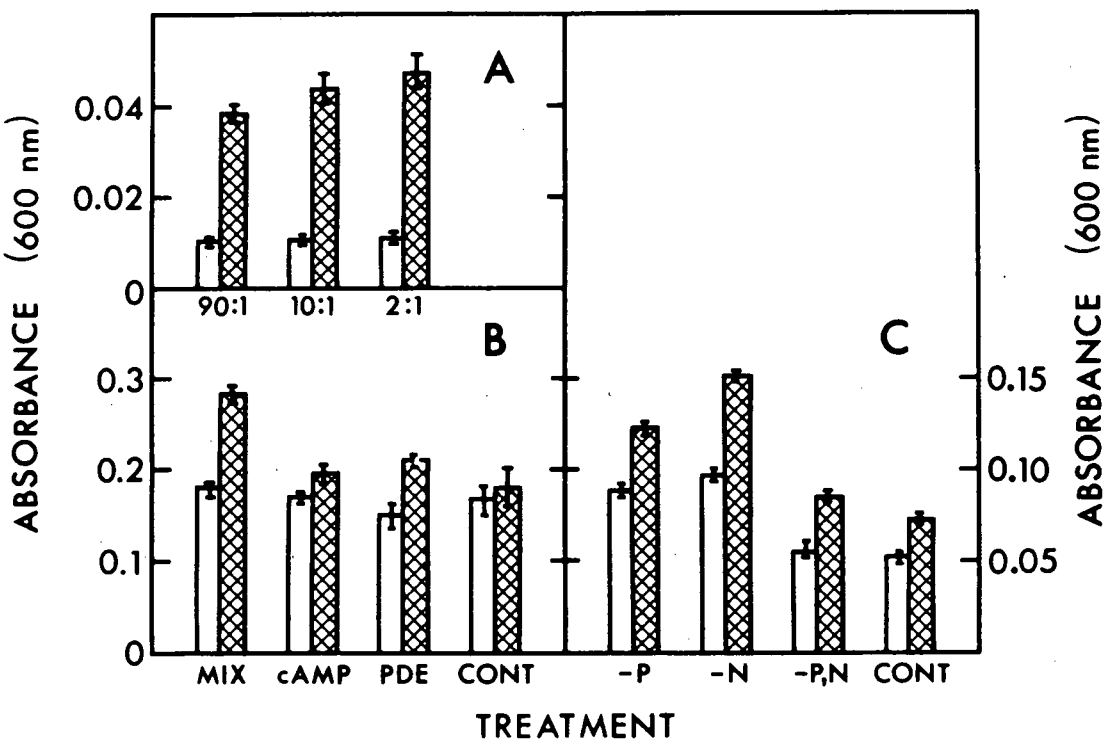

Figure 2. Growth rates as determined by changes in culture absorbance (A_{600} nm) for cultures of Anabaena flos-aquae. A) Absorbance at T_0 (open bars) and at T_7 days (hatched bars) for cultures grown under N:P ratios = 90:1, 10:1, and 2:1. B) Absorbance at T_0 (open bars) and at T_{72} h (hatched bars) for cultures containing 5 mM MIX (MIX), 1600 pmoles liter^{-1} cAMP (cAMP), 0.1 unit cyclic 3':5'-nucleotide phosphodiesterase (PDE), and normal Moss media (cont). C) Absorbance at T_0 and at T_{24} h (open and hatched bars, respectively) for cultures containing no phosphate (-P), no nitrate (-N), no phosphate or nitrate (-P, N), or normal Moss media (cont). Error bars represent ± range of duplicate cultures.

Analyses of Actively Growing Cultures of Anabaena

If cAMP is involved in some way with algal responses to nutrient limitation by nitrogen or phosphate, inoculation of cells from a nutrient-rich to a nutrient-poor medium should result in a rapid change in cellular or extracellular cAMP production. To test this hypothesis, cells were grown to late exponential phase in four batch cultures (2500-ml) containing normal Moss media. Each culture was harvested by continuous-flow centrifugation and pelleted cells from each culture were reinoculated into media containing either no P, no N, no N or P, or normal Moss media as a control. The results of these experiments are reported in Figure 3 as the means $\pm$ SD (N=2) of cellular cAMP (pmoles g^{-1} fresh wt) and extracellular cAMP (pmoles l^{-1}) of cell and filtrate samples collected immediately prior to inoculation (T_o h) and at 1 h, 8 h, and 24 h post-inoculation. Differences noted in the initial levels of cAMP in each experiment were probably due to slight differences in culture synchrony and cell densities in each 2500-ml batch culture. Each 2500-ml batch culture yielded only enough cellular material for inoculation into one series (8 samples) of each nutrient variant. The 2500-ml batch cultures were not pooled in order to inoculate each nutrient variant simultaneously because the large number of analyses done on each culture at each sampling time was prohibitive. Despite these limitations, several conclusions could be drawn.

Cells reinoculated into media deficient in P produced a 50% increase in cellular cAMP and nearly a 100% increase in extracellular cAMP within the first hour after inoculation (Figure 3a). Cellular cAMP then declined slightly 8 h and 24 h after inoculation. Extracellular cAMP declined in concentration to undetectable levels within 24 h

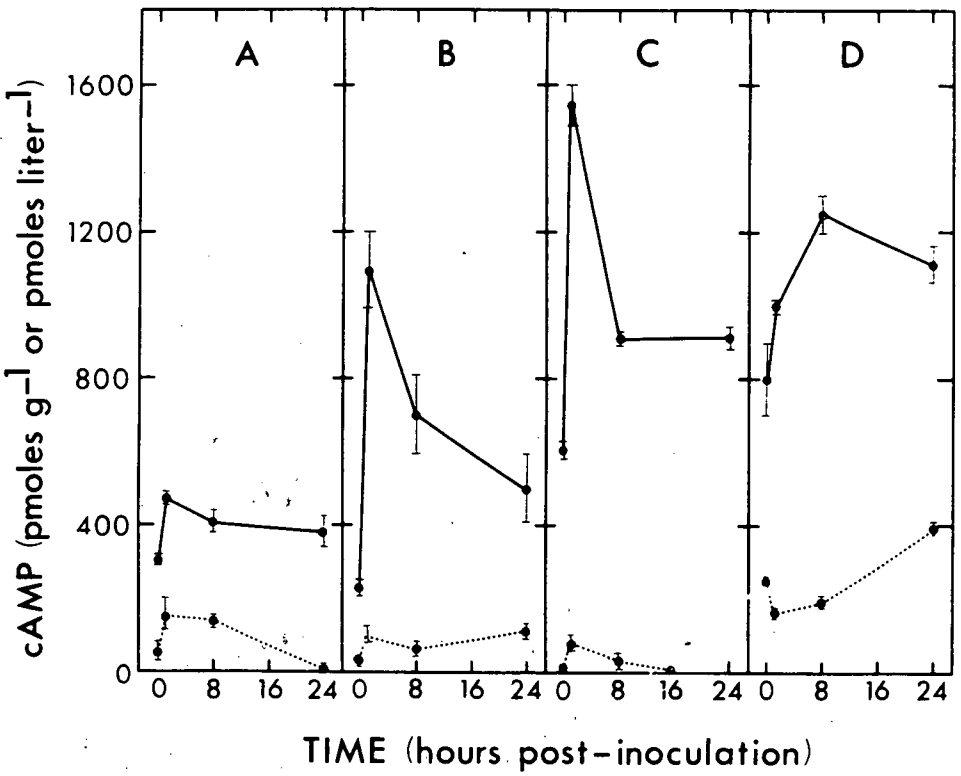

Figure 3. Cellular cAMP (——————) in pmole g^{-1} fresh wt, and extracellular cAMP (••••••••••••••) in pmoles $liter^{-1}$ in actively growing cultures of Anabaena flos-aquae inoculated into Moss media containing: A) no phosphate; B) no nitrate; C) no phosphate or nitrate; D) normal Moss media. Error bars represent mean ± 1 S.D. of replicate cultures.

post-inoculation. Alkaline phosphatase activity, undetectable at T_o-T_{8h}, was induced by 24 h post-inoculation (10 µmoles P released X 10^{-4} g^{-1} fresh wt min^{-1}).

N-limitation resulted in nearly a four-fold increase in cellular cAMP within one hour (+880 pmoles g^{-1} fresh wt) after inoculation (Figure 3b), followed by a decline in cellular cAMP levels at 8 h and 24 h post-inoculation to a level approximately 300 pmoles g^{-1} fresh wt higher than the pre-inoculation level. Heterocyst number, which had remained at about 1 heterocyst per 40 cells from T_o-T_8, increased to about 1 heterocyst per 10 cells by $T_{24\ h}$. Phosphatase activity was undetectable throughout the experimental period. Extracellular cAMP (Figure 3b) remained at low levels during the duration of the experiment.

Inoculating cells into media deficient in both N and P resulted in differential cellular cAMP production similar to that noted when N alone was limiting (Figure 3c). Cellular cAMP increased by about 950 pmoles g^{-1} fresh wt within the first hour after inoculation and declined to a level about 300 pmoles g^{-1} fresh wt higher than initial value within 24 h after inoculation. Extracellular cAMP increased slightly (Figure 3c) one hour after inoculation, but declined to an undetectable level within 24 h. Phosphatase activity, which was at undetectable levels at T_o and T_1, was induced within 8 h (2 µmoles P released X 10^{-4} g^{-1} fresh wt min^{-1}) and increased to 9 µmoles P released X 10^{-4} g^{-1} fresh wt min^{-1} at $T_{24\ h}$. Heterocyst number increased from about 1 heterocyst per 40 cells at T_o-$T_{8\ h}$ to about 1 heterocyst per 10 cells at $T_{24\ h}$.

Inoculation of cells into fresh Moss media also resulted in increased cellular cAMP production (Figure 3d). Cellular cAMP increased

by about 200 pmoles g^{-1} fresh wt within the first hour and reached a peak at 8 h post-inoculation, followed by a decline to a level approximately 300 pmoles g^{-1} fresh wt above the pre-inoculation value. Extracellular cAMP (Figure 3d) declined slightly immediately after inoculation, but then increased in concentration through the remainder of the experimental period. Phosphatase activity remained at undetectable levels for the duration of the experiment and heterocyst number declined from about 1 heterocyst per 50 cells at the beginning of the experiment to about 1 heterocyst per 100 cells at $T_{24\ h}$.

Interpretation of these results was difficult in view of the noted increases in cAMP in Moss media-containing cultures. However, the most marked increase in cellular cAMP concentration occurred when cells were inoculated into N-deficient media. This observation was consistent with results noted by Hood, et al., (1979) where N-limitation in Anabaena variabilis resulted in similarly increased cellular cAMP production. Conversely, P-limitation resulted in a less marked increase in cellular cAMP than did reinoculation into fresh Moss media, suggesting that P-limitation and the resultant induction of alkaline phosphatase activity was not related to increased cAMP production. Further, the absolute increases in cellular cAMP in the first hour in cultures deficient in N alone and N and P combined were statistically similar (880 vs. 950 p moles g^{-1} fresh wt), as were the elevated levels of cellular cAMP at the end of the experimental period as compared to pre-inoculation levels (+300 pmoles g^{-1} fresh wt), despite the fact that combined N and P-limitation resulted in more rapid induction of alkaline phosphatase activity than P-limitation alone.

The marked increase in cellular cAMP in N-limited cultures

immediately preceded heterocyst formation, suggesting that heterocyst formation in Anabaena may be regulated by intracellular cAMP, a hypothesis consistant with that proposed by Hood, et al. (1979). However, as noted, reinoculation of cells into normal Moss media also resulted in marked increases in cellular cAMP levels, suggesting that stress induced by centrifugation increased in the rate of cAMP production or that inoculation into any medium differing from that to which cells have become adapted may result in differential cAMP production.

Alternately, the observation that cAMP levels changed markedly in cultures containing adequate nutrients suggest that the levels of cellular cAMP may be highly dynamic during phases of active growth, an effect distinct from effects of differential nutrient limitation. In some mammalian cells, cAMP is known to regulate the rate of growth and cell division (Pastan, et al., 1975). The possibility that differential production of cAMP may regulate cell division in Anabaena must therefore be superimposed on the differential rates of cAMP production resulting from nutrient limitation noted in these experiments.

The dynamics of extracellular cAMP in actively growing Anabaena cultures with respect to nutrient limitation were unclear. Phosphate-limitation resulted in a decline in extracellular cAMP to undetectable levels within 24 h after inoculation into P-free media, suggesting that P-limitation inhibited cAMP release (Figure 3a,c). Extracellular cAMP levels generally increased in cultures in which cells were reinoculated into fresh Moss media, suggesting that alleviation of nutrient stress resulted in increased release of cAMP, perhaps as a means of lowering intracellular cAMP concentrations. However, cell densities had also

increased from T_0-T_{24} h, meaning that the rate of cAMP release on a per-cell basis was relatively unchanged throughout the experimental period. In actively growing cultures, however, in all nutrient variants employed and at all sampling times, most of the cAMP was found associated with cells (> 75%) rather than in extracellular form dissolved in the culture media, as opposed to stationary phase cultures of Anabaena in which the majority of cAMP was found in the extracellular media.

Chlorophyll a content, ^{14}C-bicarbonate uptake, and growth rates were measured on subsamples of each culture prior to harvest. As evidenced by culture absorbance data presented in Figure 4a-d, all cultures responded to reinoculation in fresh media by resuming log phase growth. The highest growth rate was noted in cultures inoculated into N-free media (Figure 4b). Chlorophyll a content, reported as the mean of single determinations from each duplicate culture, (Figure 4a-d) declined in the first hour after inoculation under all media conditions. In cultures containing no N and no N and P, the chlorophyll a content then remained at this reduced level for the remainder of the experimental period. In cultures deficient in P and in Moss media cultures, chlorophyll a content decreased continually for the duration of the experimental period.

The rate of ^{14}C-bicarbonate uptake, reported as the mean $\pm$ 1 S.D. (N=4) in DPM $^{14}C \times 10^8$ g^{-1} fresh wt h^{-1}, declined precipitously in P-free and P and N-free cultures within the first hour (Figure 4a,c). ^{14}C-bicarbonate uptake rates then increased concommitant with the induction of alkaline phosphatase activity.

In N-free cultures, ^{14}C-bicarbonate uptake rates remained relatively constant during the experimental period (Figure 4b). In Moss

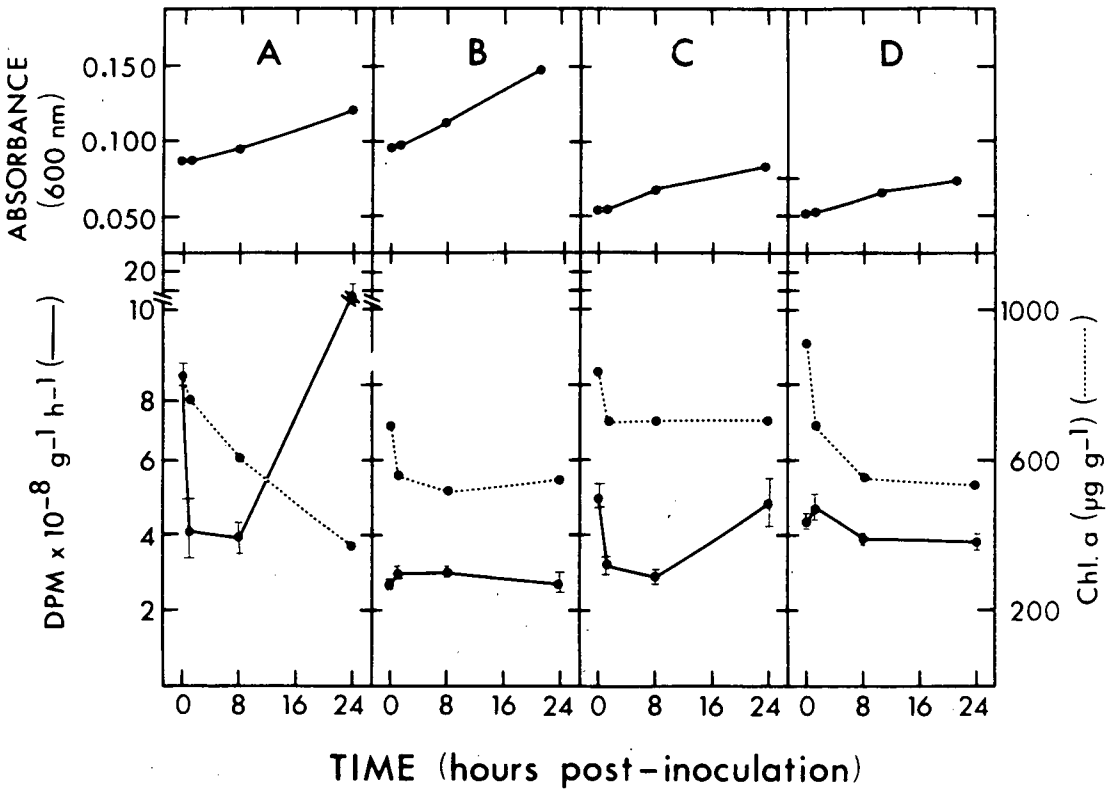

Figure 4. Dynamics of culture densities (A_{600} nm, mean of replicate cultures), chlorophyll a content (µg chlor a g^{-1} fresh wt, mean of replicate cultures), and ^{14}C-bicarbonate uptake rates (DPM × 10^{-8} g^{-1} fresh wt ± 1 S.D.; N = 4), for Anabaena inoculated into Moss media containing: A) no phosphate; B) no nitrate; C) no phosphate or nitrate; D) normal Moss media.

media cultures, the uptake rate increased slightly within one hour after inoculation, then decreased slightly to a level somewhat lower than that noted prior to inoculation (Figure 4d).

These data indicated that the most marked increase in ^{14}C-bicarbonate uptake per unit chlorophyll *a* occurred in cultures which were producing alkaline phosphatase. Correlation analyses were performed relating the concentrations of cellular and extracellular cAMP to chlorophyll *a* content, alkaline phosphatase activity, ^{14}C-bicarbonate uptake rate, or growth rate. Neither cAMP fraction could be correlated to any of the above metabolic variables in any culture regime ($P >> 0.05$), suggesting that cAMP was not directly involved in regulating these variables during log phase growth.

Effect of Inhibitors of cAMP Metabolism on cAMP Production and Release and Other Parameters in Actively Growing Anabaena

If the induction of heterocyst formation or alkaline phosphatase activity is governed by an nitrate- or phosphate-catabolite repressible mechanism in *Anabaena*, similar to glucose repression in *E. coli*, and if increased cAMP levels in *Anabaena* are involved in the removal of catabolite repression, an artificially-induced increase in intracellular cAMP should result in heterocyst formation or phosphatase induction in the presence of non-limiting quantities of nitrate or phosphate. Amrheim and Filner (1973) reported that the addition of 5 mM of the methylxanthine theophylline, a potent inhibitor of cyclic 3':5'-nucleotide phosphodiesterase, to cultures of *Chlamydomonas reinhardtii* resulted in increased levels of intracellular cAMP. Accordingly, normal Moss media was prepared containing 5 mM of the

methylxanthine MIX (1-methyl - 3 -isobutyl xanthine) prior to inoculation by Anabaena harvested from 2500 ml stock cultures as in previous experiments. An examination of Figure 5 demonstrates that pre-inoculation late exponential phase cultures contained approximately 1800 pmoles g^{-1} fresh wt cellular cAMP and 270 pmoles l^{-1} extracellular cAMP. Paradoxially, while this level of cellular cAMP coincided with increased heterocyst formation in previous experiments on log phase Anabaena, heterocyst number in these pre-inoculation cultures was only about 1 heterocyst per 30 cells. At $T_{72\ h}$, duplicate MIX-containing cultures were harvested and assayed for cAMP. MIX addition resulted in increased levels of extracellular cAMP, but not intracellular cAMP (Figure 5). MIX-containing cultures did not differ significantly from control cultures grown in normal Moss media in terms of cellular cAMP at T_{72} h.

Crystalline cAMP (1600 pmoles l^{-1}) was added to Moss media prior to inoculation of a second duplicate subsample of harvested Anabaena cells. After 72 h, most of the added cAMP remained in the extracellular media (Figure 5), but intracellular cAMP levels were significantly lower than those noted in control cultures, suggesting that the turnover time of extracellular cAMP may be very slow.

The addition of 0.1 unit of crystalline cyclic 3':5'-nucleotide phosphodiesterase (PDE) to a third duplicate subsample of harvested Anabaena should have resulted in quantitative hydrolysis of extracellular cAMP within 10 min after inoculation, based on the specific activity of this enzyme and the pH and magnesium content of the Moss media used. Instead, extracellular and intracellular cAMP levels were not significantly different at $T_{72\ h}$ than those noted in control

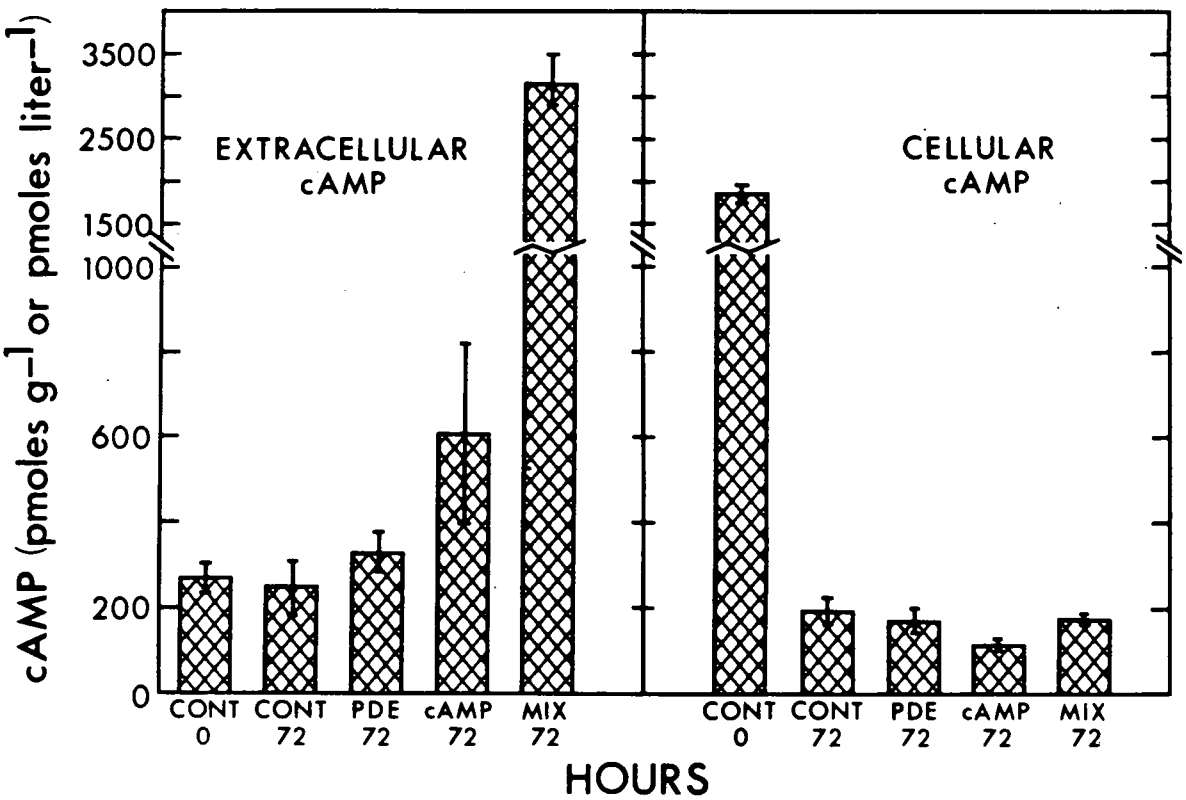

Figure 5. Effect of perturbation of cAMP levels on the dynamics of cellular cAMP (pmoles g^{-1} fresh wt) and extracellular cAMP (pmoles $liter^{-1}$) in Anabaena inoculated into Moss media containing: 5 mM MIX (MIX); 0.1 unit cyclic 3':5'-nucleotide phosphodiesterase (PDE); 1600 pmoles $liter^{-1}$ cAMP (cAMP); normal Moss media (cont). Cultures were assayed prior to inoculation and at 72 h post-inoculation. Error bars represent mean ± 1 S.D. of replicate cultures.

cultures containing no added PDE (Figure 5).

These results indicated that a natural inhibitor of PDE was produced by Anabaena, reducing the activity of added crystalline PDE. These results were consistant with previous observations (Bressan, et al., 1980a,b; Francko and Wetzel, 1980; Chapter I of this dissertation) which reported that cyclic 3':5'-nucleotide phosphodiesterase hydrolysis of putative cAMP from Ochromonas, Anabaena, Scenedesmus, and Chlamydomonas cell extracts was inhibited by an unknown compound which was removed by thin-layer chromatography. Although it was determined that Moss media itself did not inhibit PDE activity, the possibility that Anabaena released proteolytic enzymes into the media which hydrolyzed added PDE could not be ruled out.

The accumulation of extracellular rather than intracellular cAMP in MIX-containing cultures was surprising. It was possible that cAMP accumulated intracellularly during the first hours after inoculation, and was released into the media at a later time. More extensive characterization of this system will be required before experimental perturbation of intracellular cAMP levels can be used in mechanistic studies of the effects of intracellular cAMP.

Several observable differences in metabolic responses appeared to result from cAMP perturbation. Table 3 reports data on the rates of ^{14}C-bicarbonate uptake, chlorophyll a content and ratio of extracellular to cellular cAMP l^{-1} for duplicate cultures in each perturbational regime. MIX-containing cultures exhibited the highest rates of ^{14}C-bicarbonate uptake and ratios of extracellular to cellular cAMP l^{-1}, while both MIX-and exogeneous cAMP-containing cultures exhibited higher chlorophyll a contents than either PDE-containing or control cultures.

Table 3. Chlorophyll *a*, ^{14}C-bicarbonate uptake, and extracellular/cellular cAMP liter^{-1} ratio for actively growing *Anabaena* under conditions of cAMP perturbation. 1) 5 mM MIX; 2) 1600 pmoles liter^{-1} added cAMP; 3) 0.1 unit cyclic 3':5'-nucleotide phosphodiesterase; 4) normal Moss media. Chlorophyll *a* values (µg chlor *a* g^{-1} fresh wt) are given as the mean of replicate cultures, ^{14}C-uptake (DPM x 10^{-8} g^{-1} fresh wt h^{-1}) as the mean± 1 S.D.; N=4 of replicate cultures, the extracellular/cellular cAMP liter^{-1} ratio as the mean ± 1 S.D. of replicate cultures.

Treatment	Chlor *a*	^{14}C-uptake	Extracellular cAMP / Cellular
+MIX	590	4.3 ± 0.2	4.3 ± 0.4
+cAMP	570	3.7 ± 0.2	1.4 ± 0.1
+PDE	420	3.8 ± 0.4	0.59 ± 0.1
+Moss	510	3.0 ± 0.8	0.50 ± 0.1

Further, an examination of Figure 2b demonstrates that MIX-containing cultures exhibited the highest growth rate and largest standing crop biomass of all cultures examined. These results are consistant with the view that increased extracellular cAMP levels resulted in increased chlorophyll a synthesis, productivity, and growth rates in actively growing Anabaena. However, these differences may have been due to some pharmacological effect of MIX not related to cAMP metabolism.

In another experiment, the addition of MIX to early log phase Anabaena cultures resulted in the formation of large clumps of cells which could not be disrupted even by vigorous agitation. When clumped cells were centrifuged and resuspended in Moss media without MIX, the clumps dissociated within 4 h. The rate of dissociation of clumps was not hastened by the addition of cyclic 3':5'-nucleotide phosphodiesterase to resuspended cells, nor did the addition of large amounts (2000 pmoles l^{-1}) of exogeneous cAMP prevent clumps from dissociating in Moss media deficient in MIX. These results suggested that MIX may have pharmacological effects not related to cAMP metabolism in Anabaena. In addition, the indication of clumping behavior by MIX was apparently restricted to log phase cells; when 5 mM MIX was added to stationary phase cultures of Anabaena, no clumping behavior was noted.

CONCLUSIONS

The production and extracellular release of cAMP by Anabaena varied greatly during the life cycle of the organism and under differing nutrient regimes. An increased production of intracellular cAMP by Anabaena in N-free media immediately preceded heterocyst formation, but the data did not conclusively demonstrate that cAMP was actively

involved in the induction process. Conversely, alkaline phosphatase induction did not seem to be correlated to changes in intracellular or extracellular cAMP, although phosphate limitation resulted in a marked decrease in extracellular cAMP release.

The addition of MIX to actively growing cultures of Anabaena resulted in marked extracellular but not intracellular cAMP accumulation, the first time a prokaryotic organism has been shown to contain a methylxanthine-sensitive cyclic 3':5'-nucleotide phosphodiesterase. The evidence indicated that the noted MIX-induced increased levels of extracellular cAMP resulted in increased growth rates, ^{14}C-bicarbonate uptake and chlorophyll a synthesis, and that Anabaena produced an exogeneous inhibitor of cyclic 3':5'-nucleotide phosphodiesterase, although proteolytic degradation of added diesterase could not be discounted. Concentrations of intracellular cAMP could be significantly correlated to ^{14}C-bicarbonate uptake rates by an exponential relationship in stationary phase Anabaena cultures, suggesting that cAMP may be involved in release of catabolite repression mechanisms operating in photosynthesis. Significant correlations could not be determined for the relationships of cellular or extracellular cAMP to chlorophyll a synthesis, ^{14}C-bicarbonate uptake or alkaline phosphatase induction in actively growing or stationary phase cultures of Anabaena.

In stationary phase cultures of Anabaena most of the cAMP existed in extracellular form dissolved in the media, while most of the cAMP actively growing cultures was associated with cells. These data indicated that the relative rates of cAMP release from Anabaena cells may be a function of the rate of population increase as well as nutrient

dynamic behavior.

Collectively, these data indicate that cAMP may serve several different functions in Anabaena. The observation that cellular and extracellular cAMP levels varied markedly between active growth phase stationary phase in in cultures containing the same nutrient regimes suggest that cAMP may have functions in the synchronization of the cell cycle, similar to those noted in other organisms (Pastan, et al., 1975). The relationship between the dynamics of cAMP and nutrient dynamic behavior and primary productivity suggest that cAMP may also be involved in the regulation of homeostatic responses under changing environmental conditions in this algal species.

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CHAPTER III

THE ISOLATION OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE (cAMP) FROM LAKES OF DIFFERING TROPHIC STATUS: PHYSIOLOGICAL AND ECOLOGICAL IMPLICATIONS

INTRODUCTION

Cyclic adenosine 3':5'-monophosphate (cAMP) is an important metabolic regulatory molecule in a wide variety of prokaryotic and eukaryotic organisms. Recent studies have shown that the cultured phytoplankton species Chlamydomonas reinhardtii (Amrhein and Filner, 1973; Bressan, et al., 1980b), Ochromonas malhamensis (Bressan, et al., 1980a), Anabaena variabilis (Hood, et al., 1979), and eight other species of green and blue-green algae (Francko and Wetzel, 1980) produce variable quantities of cAMP and release it extracellularly into the medium.

The occurrence of cAMP in natural lacustrine systems has not been investigated. On the basis of studies on cultured phytoplankton and bacteria, it is reasonable to suggest that cAMP may exist in lakes associated with plankton and dissolved in lakewater. Firm evidence that cAMP exists in aquatic systems is a necessary prelude to studies on the seasonal dynamics and potential ecological significance of cAMP these systems.

In many bacteria and two phytoplankton species studied to date (Hood, et al., 1979; Bressan, et al., 1980a), increased cAMP production and extracellular release have been correlated to the onset of nutritional stress imposed by carbon- or nitrogen-limiting conditions. In bacteria, cAMP operates as a secondary messenger involved in the

removal of catabolite repression to nitrogen and carbon substrates. An analogous role has been suggested, but not substantiated, for cAMP in photosynthetic organisms.

Although it is known that phytoplankton and other aquatic organisms modify their metabolic responses to changing environmental conditions, little is known of the molecular mechanisms controlling these changes. For example, it is known that phosphate limitation induces the production of alkaline phosphatase in many cultured phytoplankton and in natural aquatic communities (Kuenzler and Perras, 1965; Heath and Cooke, 1975). Similarly, some blue-green algae are capable of heterocyst formation in response to combined nitrogen limitation (Wolk, 1973). The dynamic nature of chlorophyll synthesis, primary productivity, and growth rates during the life cycles of photosynthetic organisms are also examples of metabolic plasticity. In each case, the actual sequence of events from environmental stimulus to the noted response is poorly understood.

These inducible responses to environmental stress are examples of homeostatic control mechanisms, which increase in importance as direct organismal control over resources increases (Margalef, 1968; Odum, 1969). In the complex network design of aquatic communities, many molecular "fine-tuning" mechanisms have likely evolved which regulate aspects of population dynamics and community interactions.

This study presents evidence, on the basis of several analytical criteria, that particulate and dissolved cAMP occur in two hardwater lakes of differing trophic status, and preliminary evidence that differential production and extracellular release of cAMP by epilimnetic plankton communities may be involved in community dynamics, primary

productivity regulation, chlorophyll synthesis and alkaline phosphatase activity in these systems.

MATERIALS AND METHODS

Collection and Processing of Samples for Assay of cAMP

Epilimnetic lakewater samples (20-90 liters) were collected at mid-day from the 0.1-m strata of Lawrence Lake, a hardwater, oligotrophic lake and Wintergreen Lake, a hardwater, hypereutrophic lake, both in southwestern Michigan, at sampling stations located over the deepest depressions of each basin (12.6 m and 6.5 m, respectively), and within the littoral zones of each lake. Particulate matter present in each lakewater sample was harvested on a Sorvall model SS-3 continuous-flow centrifuge at 10,000 g using a flow-through rate of 180 ml min^{-1}. Filtration of effluent water and microscopic examination of pellet aliquots demonstrated that this sedimentation force quantitatively removed (> 90%) suspended particulate matter without rupturing cells. Fresh weight of pelleted material was determined on a Mettler Model E200 balance.

Centrifuged particulate matter was extracted in 0.5 N $HClO_4$ (1 ml 0.1 g^{-1}) at 0°C, purified (Bressan, et al., 1980a,b; Handa and Bressan, 1980), and redissolved in 50 mM Tris-HCl, pH 6.8.

Lakewater filtrate samples were collected by gently filtering 250-ml aliquots of each culture through pre-washed 0.5-µm pore size Reeve-Angel (984H) glass-fiber filters using a vacuum differential of 0.5 atm.

Dissolved cAMP present in lakewater filtrate samples was recovered by adsorption and elution from purified (Handa and Johri, 1977) Norit A

and was redissolved in 50 mM Tris-HCl, pH 6.8 (Bressan, et al., 1980a,b). All samples in 50 mM Tris-HCl were further purified by neutral alumina and Dowex 50 chromatography, lyophilized, redissolved in distilled, deionized water (Bressan, et al., 1980a,b), and assayed for cAMP. Radioactivity was measured in Insta-Gel (Packard Co.) scintillation fluid on a Beckman model 8000 liquid scintillation counter. Recoveries of ^{3}H-cAMP internal standard in both cell and filtrate samples varied from 5-30%.

Cyclic AMP binding protein, isolated from bovine heart (Kuo and Greengard, 1972), was used to assay cAMP by the Gilman protein binding assay (Gilman, 1972). Samples were boiled for 3 min. before assaying to denature interfering substances present even after extensive purification (R. A. Bressan, personal communication). Contamination by cAMP present in glassware and reagents never exceeded the detectability limits of the assay (0.01 pmoles assay tube^{-1}). Cyclic AMP-dependent protein kinase from bovine heart was used to measure cAMP by the method of Handa and Bressan (1978, 1980). The amount of ATP remaining in each reaction mixture was measured by the firefly luciferin-luciferase system. Luminescence was measured with an ATP photometer (Aminco 4-7441) coupled to a digital peak integrator (Columbia Scientific Industries 208). Particulate cAMP was expressed as pmoles g^{-1} fresh wt while dissolved cAMP was expressed as pmoles liter^{-1} filtered lakewater.

The kinetics of cyclic 3':5'-nucleotide phosphodiesterase hydrolysis of cAMP measured by the Gilman assay were determined according to Handa and Johri (1977) and Bressan, et al. (1980a,b). Lyophilized Dowex 50 samples were chromatographed by silica gel thin-layer chromatography. The resulting cAMP fraction (R_f = 0.75) was

digested with phosphodiesterase. Controls containing theophylline, (1, 3-dimethyl xanthine), a specific inhibitor of cyclic 3':5'-nucleotide phosphodiesterase, were measured concurrently.

Determination of Chlorophyll a Content, Primary Productivity, and Alkaline Phosphatase Activity in Lakewater Samples.

Subsamples (100-1500 ml) of lakewater collected for cAMP analysis were filtered through 0.8-μm pore size Millipore (AA) filters using a vacuum differential of 0.5 atm. Chlorophyll a content of each filter, corrected for phaeophytin, was measured spectrophotometrically by the trichromatic method (Wetzel and Likens, 1979) and converted to units of μg chlorophyll a g^{-1} fresh wt.

Primary productivity in Lawrence Lake was assayed in situ at 0.1-m and 1-m depths by the ^{14}C-bicarbonate uptake method (Wetzel and Likens, 1979). Light and dark bottles (125 ml) were suspended for 4-h periods during midday and ^{14}C-incorporation was converted to mg C g^{-1} fresh wt day^{-1}.

Alkaline phosphatase activity was assayed on 0.5 ml subsamples of lakewater samples prior to centrifugation by the fluorometric assay of Kuenzler and Perras (1965). Total phosphatase activity was corrected for soluble phosphatase activity and is expressed as moles particulate phosphatase activity in μmoles P released X 10^4 g^{-1} fresh wt min^{-1}.

RESULTS AND DISCUSSION

The Characterization of Putative cAMP from Lakewater Samples

Complete characterization of putative cAMP from any source which has not previously been investigated is imperative. The majority of

papers on the supposed existence and function of cAMP in photosynthetic organisms have been rendered unconvincing by incomplete characterization of cAMP-like material. Bressan, et al. (1976) demonstrated that plants possess interferring substances which produce positive but erroneous results in the commonly used Gilman protein binding assay and the protein kinase assay for cAMP. Recent work has demonstrated (Bressan, et al., 1980 a,b; Handa and Bressan, 1980; Francko and Wetzel, 1980; Chapter I of this dissertation) that these assays can be used to assay cAMP from phytoplanktonic organisms provided that samples are purified with neutral alumina and Dowex 50 chromatography prior to assay.

Accordingly, several independent analytical techniques were utilized to demonstrate that authentic particulate and dissolved cAMP were present in lakewater samples. Samples collected from Lawrence Lake and Wintergreen Lake were purified and assayed by the Gilman protein binding assay, the protein kinase luciferin-luciferase assay, and the cyclic 3':5'-nucleotide phosphodiesterase kinetics assay.

Each particulate and lakewater fraction contained a factor which co-purified with authentic cAMP in neutral alumina, Dowex 50, and silica gel chromatography, replaced authentic cAMP in the Gilman and protein kinase assays, and which was degraded by cyclic 3':5'-nucleotide phosphodiesterase at the same rate as authentic cAMP. Particulate and dissolved cAMP levels were measured equally well by either the protein kinase assay or the Gilman assay (Table 1).

Aliquots of purified particulate and filtrate samples were augmented with authentic cAMP (0.5 pmoles assay tube^{-1} in samples containing from 0.6-3.0 pmoles assay tube^{-1}) before analysis by the Gilman assay to demonstrate that non-specific interference did not

Table 1. Comparison of particulate and dissolved cAMP in Wintergreen Lake and Lawrence Lake samples as determined by the Gilman protein binding assay and the protein kinase luciferin-luciferase (PKL) assay. Values in parentheses indicate + % error in the predicted concentration of cAMP when samples (0.6 - 3.0 pmoles assay tube^{-1}) were augmented with 0.5 pmoles of authentic cAMP prior to analysis by the Gilman assay. All values are in units of pmoles g^{-1} fresh wt (particulate) or pmoles l^{-1} (filtrate) ± 1SD.

	1979	PKL Assay		Gilman Assay	
Lake	Date	Particulate	Filtrate	Particulate	Filtrate
Lawrence	1 May	203.8±40	53.1±11	195± 10(+6%)	39.8±4(-5%)
	8 May	385.1±80	133.5±26	365.1±18	146.5±8
	23 May	473.3±95	16.4±4	493.2±25	16.4±2
	30 May	600±120	295.3±60	600±30(-5%)	324.5±17(+9%)
Winter-green	1 May	239.4±47	183.6±36	257.5±13(+9%)	168.2±8(+5%)
	8 May	91.9±18	125±24	110.9±5	155.4±8
	23 May	219.9±44	178.8±35	238.9±12	177,2±10
	30 May	596.9±120	90.8±18	575±30(+8%)	101±5(-5%)

occur. Additive cAMP levels in both particulate and filtrate samples varied < 10% from predicted values, further evidence that the Gilman assay measured authentic cAMP (Table 1).

When purified particulate and filtrate samples from Wintergreen Lake and Lawrence Lake were incubated with cyclic 3':5'-nucleotide phosphodiesterase, protein binding activity was abolished at the same rate as with authentic cAMP (Figure 1). Furthermore, theophylline completely inhibited diesterase hydrolysis of both authentic cAMP and putative cAMP from the same lake samples (Figure 1).

Collectively, the evidence demonstrated that authentic cAMP was present in lakewater from two trophically dissimilar lakes. These data also demonstrated that the Gilman assay was suitable for the assay of cAMP from aquatic systems, provided that samples were purified with neutral alumina and Dowex 50 chromatography. In view of the relative simplicity and precision of this assay, it was used for the remainder of cAMP determinations in this paper.

Seasonal Dynamics of Particulate and Dissolved cAMP in Two Lakes of Differing Trophic Status.

If cAMP is involved in some aspects of trophic dynamic regulation in aquatic systems, and would anticipate that the concentrations of dissolved and particulate cAMP would vary dynamically, both seasonally and between lakes of differing trophic status, in response to differing environmental conditions. When the concentrations of cAMP present in the 0.1-m stratum of the epilimnia of Wintergreen Lake and Lawrence Lake were compared on a seasonal basis, considerable temporal variation was noted in the dissolved and particulate cAMP concentrations found in each lake (Figures 2 and 3). Further, particulate and dissolved cAMP in

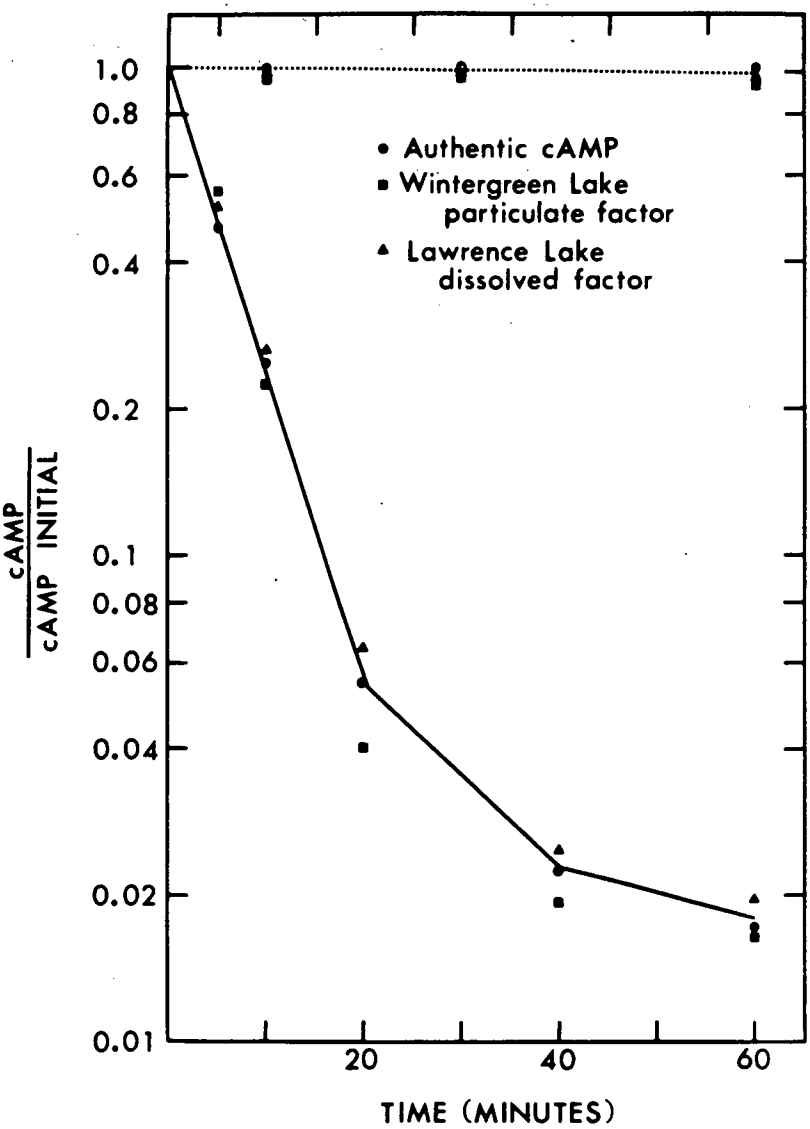

Figure 1. Kinetics of cyclic 3':5'-nucleotide phosphodiesterase hydrolysis of authentic cAMP and putative cAMP isolated from pelleted Wintergreen Lake particulate matter and from filtered Lawrence Lake water (——————). Shown is the fraction of original cAMP remaining in the reaction mixture as a function of incubation time. Hydrolysis kinetics in the presence of 5 mM theophylline (••••••••••••••). All values were determined by the Gilman assay.

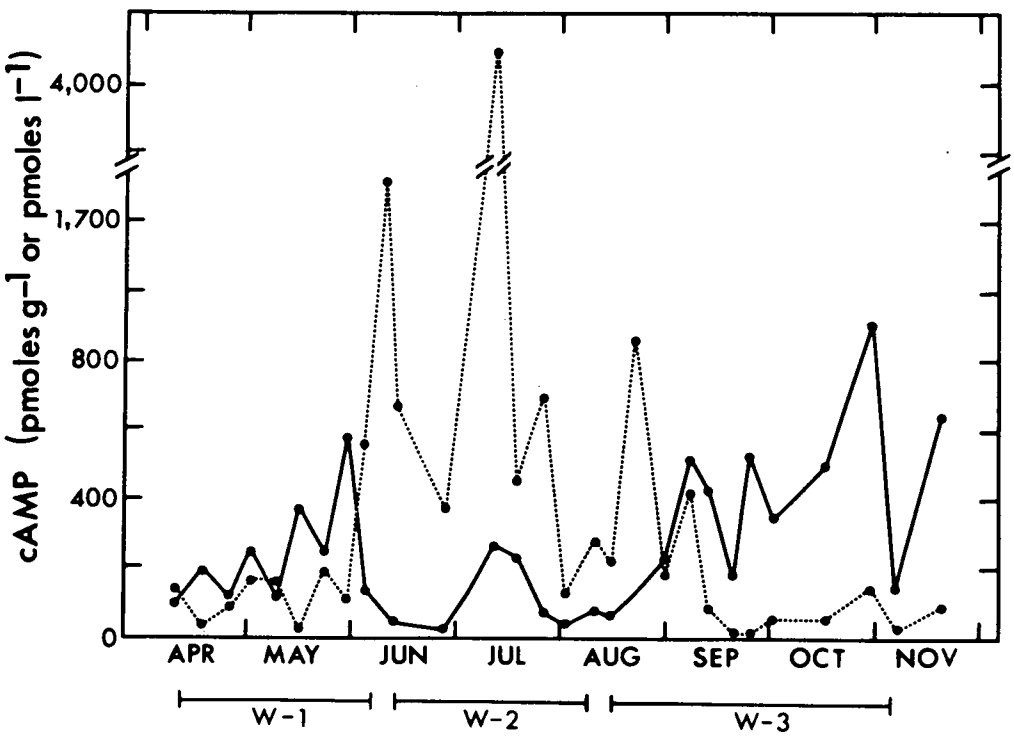

Figure 2. Particulate cAMP (——————) in pmoles g^{-1} fresh wt, and dissolved cAMP (••••••••••••••) in pmoles $liter^{-1}$, for epilimnetic samples from Wintergreen Lake, 1979, as determined by the Gilman assay. W-1, W-2, and W-3 denote durations of phytoplankton associations described in Table 2.

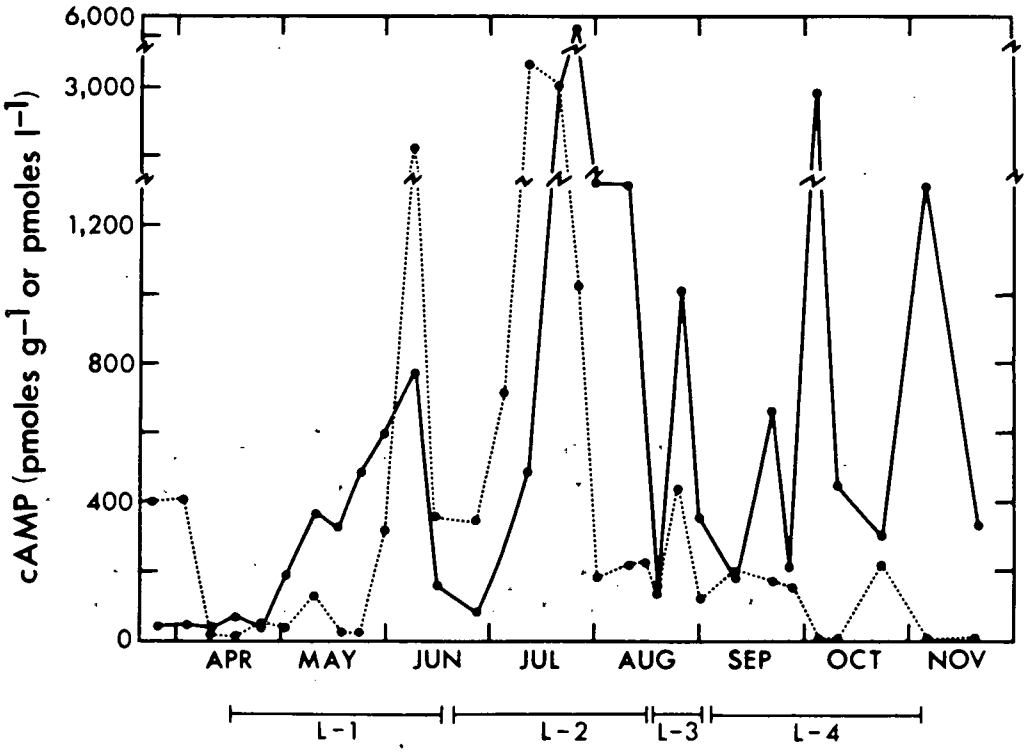

Figure 3. Particulate cAMP (——————) in pmoles g^{-1} fresh wt, and dissolved cAMP (·····) in pmoles $liter^{-1}$, for epilimnetic samples from Lawrence Lake, 1979, as determined by the Gilman assay. L-1, L-2, L-3, and L-4 denote durations of phytoplankton associations described in Table 2.

samples collected the same day from each lake differed significantly between the two lakes. Particulate biomass estimates were derived from fresh weight determinations, a factor necessitated by subsequent purification procedures. Estimates of fresh weight were subject to an error of $\pm$ 15% ($\pm$ 1 SD; N = 3), while the Gilman assay values for cAMP were subject to an error of $\pm$ 5% ($\pm$ 1 SD; N = 5) in a range from 0.05-4 pmoles assay tube^{-1}. The determination of dissolved cAMP concentrations was subject only to the variance of the Gilman assay itself. From these considerations, the noted temporal and interlake differences in cAMP concentrations resulted from dynamic processes occurring within the planktonic community and not sampling or analytical error.

Analysis of aliquots of pelleted particulate matter indicated that the volumetric ratio of phytoplankton:bacteria:detritus was generally about 5:1:1 in Lawrence Lake and 4:1:1 in Wintergreen Lake during April-November 1979. Preliminary examination of the cAMP production of two aquatic bacteria isolated from Lawrence Lake indicated cAMP production and release comparable to that noted in cultured phytoplankton (Francko and Wetzel, unpublished data). Although the production of cAMP by zooplankton has not been investigated, they comprised < 5% of the total pellet biomass during the study period. The evidence indicated that the noted levels of cAMP were largely the result of phytoplanktonic activity. It should be noted, however, that fresh weight determinations of biomass, coupled with the presence of differential amounts of particulate detrital matter during the year may have resulted in slight over- or underestimates of particulate-associated cAMP in each lake on any given sampling date.

These considerations not withstanding, several inferences can be

drawn from the seasonal dynamics of cAMP presented in Figures 2 and 3. It should be emphasized that particulate cAMP was reported in pmoles g^{-1} biomass, while dissolved cAMP was reported in units of pmoles $liter^{-1}$ filtered lakewater. Thus, an increase in particulate cAMP concentration represented a relative increase in the amount of cAMP per organism while dissolved cAMP was a relative measure of the release of cAMP from planktonic organisms and potential inputs of dissolved cAMP from littoral and allochthonous sources.

In Wintergreen Lake (Figure 2), particulate cAMP levels generally increased during the early spring period following the loss of ice cover in late March, reaching a maximum level in late May. Another increase in particulate cAMP was noted in midsummer, while the third period of increase occurred in late summer and fall, transcending fall circulation, which occurred in this lake in early October. Dissolved cAMP levels were generally low in the spring and fall, while the maximum concentrations of dissolved cAMP occurred in midsummer.

In Lawrence Lake (Figure 3), a similar pattern of seasonal dynamics with regard to particulate and dissolved cAMP was observed. However, the absolute amounts of both cAMP fractions were generally much higher in Lawrence Lake during all seasons of the year. Further, a higher degree of synchrony in peaks of dissolved and particulate cAMP concentrations occurred in Lawrence Lake than in Wintergreen Lake, particularly in the late spring and mid-summer periods. Although extensive winter sampling was not conducted, samples collected soon after ice cover was established in December and about two weeks before loss of ice cover the following spring, suggested that winter phytoplankton populations in both lakes produced and released

approximately the same amount of cAMP reported for mid-April samples in Figures 2 and 3.

The phytoplankton density in hypereutrophic Wintergreen Lake was generally about an order of magnitude greater than that found in oligotrophic Lawrence Lake. Thus, seasonal differences in the amount of cAMP per g biomass or the amount of cAMP per liter as represented in Figures 2 and 3 may be misleading. Accordingly, the data presented in Figures 2 and 3 was transformed into units of particulate cAMP l^{-1} and dissolved $cAMP^{-1}$. The ratio of dissolved to particulate cAMP l^{-1} (Figure 4) may be used as a measure of the relative rate of extracellular release of cAMP per organism, and the relative proportion of particulate and dissolved cAMP per liter of whole lakewater. From these data, it was evident that the highest rates of release per organism occurred in both lakes during early to midsummer. During the spring and fall, the relative rate of release was generally much higher in Lawrence Lake than in Wintergreen Lake.

Correlation of Seasonal Dynamics of cAMP with Changes in Phytoplankton Community Structure.

In mammalian cells, cAMP functions in the regulation of cell proliferation and growth rate dynamics (Pastan, et al., 1975). In phytoplankton, the production and extracellular release of cAMP appears to vary greatly during the life cycle of a given species (Chapter II) and between different species even at the same stage in growth and under similar environmental conditions (Francko and Wetzel, 1980; Chapters I & II). It may be possible, then, to ascribe the noted dynamic changes in particulate and dissolved cAMP in Lawrence Lake and Wintergreen Lake to changes in phytoplankton community structure resulting from seasonal

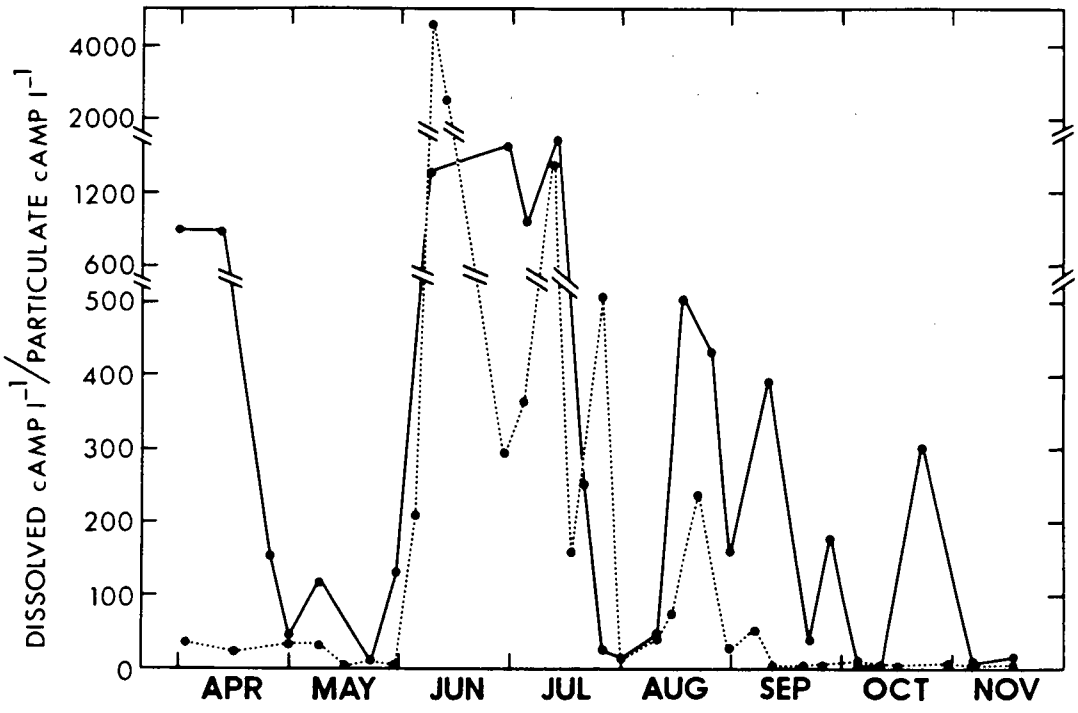

Figure 4. The ratio of dissolved to particulate cAMP in pmoles liter^{-1} whole lakewater for Lawrence Lake (——) and Wintergreen Lake (·····), 1979, as determined by the Gilman assay.

succession. In this study, no rigorous attempt was made to quantify species variety or equitability during the season. Instead, aliquots of pelleted particulate material on each sample date were examined microscopically to determine changes in dominant phytoplankton species through the year in each lake. In this manner, major phytoplanktonic associations could be delimited in each lake during the study period. These data are summarized in Table 2, and Figures 2 and 3. The relative composition of each lakewater sample determined by examination of centrifugation-pelleted material agreed with that determined by standard sedimentation chamber techniques. Data on changes in the relative abundance of phytoplankton during the development of each association, and the seasonal succession of phytoplankton associations were correlated to the dynamic changes in particulate and dissolved cAMP in each lake.

In Wintergreen Lake, three phytoplanktonic associations could be recognized during the study period: 1) a spring population comprised equally of green and blue-green algae and pennate diatoms; 2) an early to midsummer population comprised mainly of filamentous and colonial green algae and dinoflagellates, and; 3) a late summer-fall population consisting of non-heterocystous blue-green algae, dinoflagellates, green algae and pennate diatoms.

An examination of Figure 5 demonstrates that the relative changes in biomass of each of this associations were reflected in differences in pelletable particulate material during the season, an observation corroborated by phytoplankton enumeration determinations (Crumpton and Wetzel, 1980). If changes in pelletable particulate material are assumed to correspond with changes in planktonic density, an examination

Table 2

PHYTOPLANKTON ASSOCIATIONS IN LAWRENCE LAKE AND WINTERGREEN LAKE, 1979

Date	Association	Wintergreen Lake
April-early June	W-1	Mixed green and blue-green with some pennate diatoms and bacteria. Scenedesmus, Ankistrodesmus, Clorococcum, Aphanazomenon, Synedra, Pediastrum, Chlamydomonas.
Mid June-early Aug	W-2	Filamentous greens, dinoflagellates, colonial greens, microflagellates. Oedogonium, Ceratium, various chlorococcales. Oedogomium bloom covers > 50% of surface area of lake.
Early Aug-Nov	W-3	Primarily non-heterocystis blue-green algae, a few dinoflagellates and chlorococcales sp., and pennate diatoms, Microcystis, Merismopoedia, Ankistrodesmus

Date	Association	Lawrence Lake
April-early June	L-1	Cyclotella dominant, with a few cryptomonads and green algae.
Mid June-Mid Aug	L-2	Cyclotella dominant along with some microflagellates.
Mid Aug-late Aug	L-3	Large colonial greens and Dinobryon. Oocytis, some Anabaena.
Early Sept-Nov	L-4	Heterogeneous populations. Colonial greens, microflagellates, filamentous and non-filamentous blue-green algae, pennate and centric diatoms.

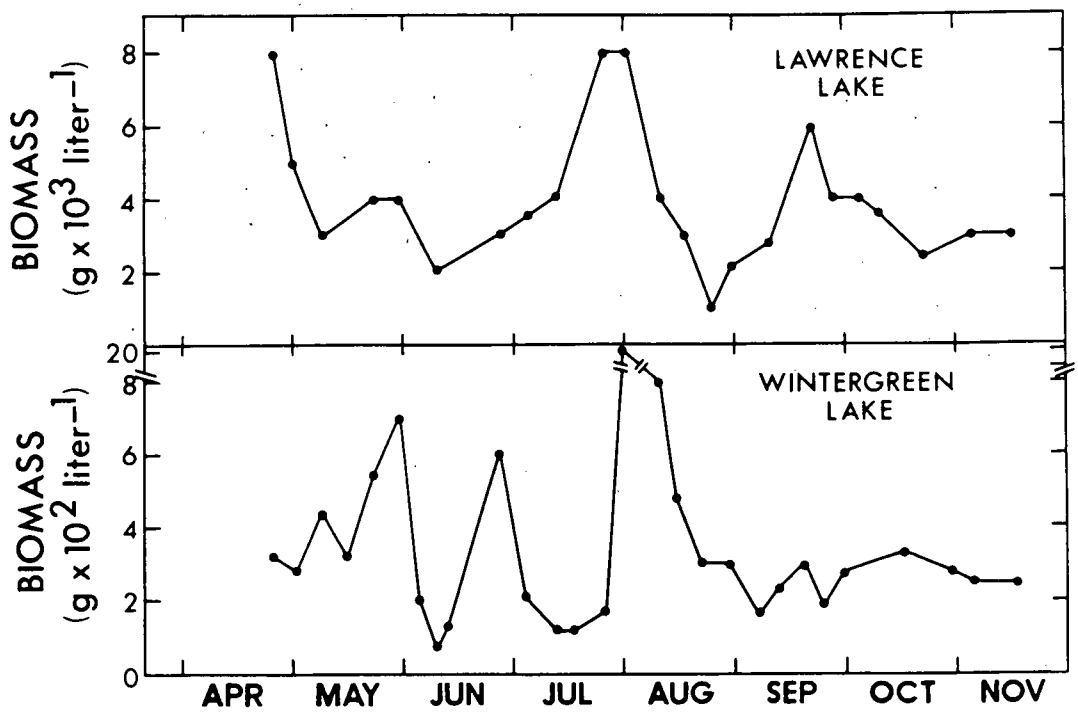

Figure 5. Pellet biomass for epilimnetic samples from Lawrence Lake ($g \times 10^3$ liter^{-1}) and Wintergreen Lake ($g \times 10^2$ liter^{-1}), 1979, reported as the mean of three determinations.

of Table 2 and Figures 2 and 5 demonstrates that the spring increase in particulate cAMP per unit biomass corresponded with an increase in plankton density. Further, the rapid decline of the spring population could be correlated with the precipitous reduction in particulate cAMP and concommitant increase in dissolved cAMP noted in early June. The increase in particulate cAMP noted between late June and late July coresponded to a decline in the early midsummer association, while the third period of particulate cAMP increase corresponded with a decline in the size of the late-summer-fall associations.

Between late June and mid-September, a bloom of Oedogonium covered approximately 50% of the surface area of the littoral and pelagial zones of Wintergreen lake. While cAMP-like material from Oedogonium was not fully characterized, Gilman assay of cellular cAMP on samples collected at the height of the bloom demonstrated that this alga contained 30 pmoles g^{-1} cellular cAMP. Because of the massive population of Oedogonium present during late June to mid-September, it is reasonable to suggest that the high dissolved cAMP levels noted during the summer in Wintergreen Lake may have resulted in part from release of cAMP by Oedogonium.

In Lawrence Lake, four major planktonic associations were present during the study period: 1) a spring association comprised of two species of Cyclotella with smaller populations of cryptomonads and green algae; 2) an early to midsummer association which was essentially a monoculture of Cyclotella species with some microflagellates; 3) a mid- to late August association comprised of large colonial green algae and Dinobryon and; 4) a late summer-fall association comprised of a heterogeneous population of colonial green algae, microflagellates,

filamentous and non-filamentous blue-green algae and pennate and centric diatoms, including Cyclotella.

An examination of both Table 2 and Figures 3 and 5 demonstrates that the spring algal population increase corresponded with an increase in particulate cAMP. The decline of the spring population during June corresponded with a decrease in particulate cAMP, while the increase in the early to midsummer population of Cyclotella corresponded to the massive increase in particulate and dissolved cAMP noted from late June to mid August. The decline of Cyclotella and subsequent development of August populations of green algae and Dinobryon were also reflected in a similar decline and increase in particulate and dissolved cAMP. Similarly, the decline of the green algal-Dinobryon association and the subsequent development of the late summer-fall association were reflected in a decline and subsequent increase in particulate cAMP.

From these data, a functional relationship between cellular and dissolved cAMP dynamics and the dynamics of seasonal phytoplanktonic succession could be inferred, but not proved. It appeared, however, that as a dominant population of algae increased in density, presumably becoming progressively more nutrient limited, the cellular production of cAMP increased, suggesting that cAMP functioned in some way as an indicator of nutrient stress in these systems.

Comparison of Pelagic and Littoral Production of cAMP and Allochthonous Inputs of cAMP.

Since the littoral zone of small lakes is often extensive and dominates the overall ecosystem metabolism of many lakes (Wetzel, 1975, 1979), the production of cAMP by organisms of the littoral community and its subsequent export to the pelagial zone of a lake may be important.

Both Wintergreen Lake and Lawrence Lake contain extensive littoral vegetation. Thus, a preliminary investigation into differential production of cAMP within the littoral and pelagial zones of each lake was conducted and is presented in Table 3. Water samples were collected in a stand of Scirpus subterminalis, the dominant submersed macrophyte in Lawrence Lake (Rich, et al., 1971) and within an extensive bed of Nuphar advena and Ceratophyllum demersum in Wintergreen Lake. Particulate cAMP levels, representing cAMP in littoral plankton, were much higher than levels noted in epilimnetic plankton sampled on the same day. Dissolved cAMP, which presumably could be the combined result of release by littoral phytoplankton, aquatic macrophytes and epiphytes, varied between littoral and pelagic samples. Extensive characterization of putative cAMP from tissues of aquatic macrophytes was not conducted, but neutral alumnia and Dowex 50-purified samples of Potamogeton zosteriformis and Cerotophyllum demersum, two macrophytes common to these systems, contained approximately 30 pmoles of cAMP g^{-1} fresh wt. as determined by the Gilman assay, suggesting that macrophytic production of cAMP may have contributed significantly to the dissolved cAMP pool present in the open waters of these lakes.

The allochthonous inputs of dissolved cAMP to these systems was also examined. Samples collected during midsummer (10 and 17 August) from the main inflow stream to Lawrence Lake, which drains an extensive marsh system, contained 229 and 79 pmoles l^{-} of dissolved cAMP, respectively.

Collectively, these data indicated that significant differences existed in dissolved and particulate cAMP production between littoral and pelagial zones of each lake. Further, significant allochthonous

Table 3. Comparison of particulate cAMP (pmoles g^{-1} fresh weight) and dissolved cAMP (pmoles $liter^{-1}$) in littoral and epilimnetic samples from Wintergreen and Lawrence Lakes, 1979.

Lake	Date	Littoral		Pelagial	
		Particulate	Dissolved	Particulate	Dissolved
Wintergreen	14 Aug.	6165	193	55	222
	12 Sept.	-	138	-	85
	1 Oct.	-	< 5	-	57
Lawrence	20 July	8754	3900	2876	1950
	17 Aug.	-	131	-	210
	21 Sept.	-	336	-	171

inputs of cAMP, though stream inflow occurred in the Lawrence Lake system.

Correlation of Particulate and Dissolved cAMP with the Dynamics of Chlorophyll a, Primary Productivity, and Alkaline Phosphatase Activity.

If the differential production and release of cAMP by members of the planktonic community of lakes was related in some way to growth and development under changing environmental conditions, cAMP dynamics should have been related to some physiological changes in these organisms. In view of the large number of potentially important interactions known to involve cAMP in heterotrophic organisms, and the paucity of data on the physiological importance of cAMP in photosynthetic organisms, some basic assumptions were made in this investigation.

If cAMP was involved in some mechanism which enabled phytoplankton to fix carbon more efficiently, whether that mechanism was directly involved in photosynthesis or not, an examination of the rates of primary productivity in situ and their correlation with the dynamics of cAMP should show a definite relationship. Further, changes in pigment concentration per cell should also have been related in some way to differential production and release of cAMP if cAMP was directly involved in pigment dynamics. If cAMP was involved in some way with physiological adaptations to nutrient limitation by phosphorus or nitrogen, a correlation should exist between the induction of alkaline phosphatase activity or heterocyst formation and the dynamics of cAMP.

Preliminary evidence from cultured phytoplankton suggests that the aforementioned assumptions may be applicable to natural systems. The differential production and release of cAMP by Anabaena sp. has been

correlated to nitrate limitation and the production of heterocysts (Hood, et al., 1979; Chapter II), chlorophyll synthesis in green (Berchtold and Bachofen, 1977) and blue-green (Chapter II) algae, ^{14}C-bicarbonate uptake in Anabaena (Chapter II) and changes in carbon substrates present in growth media in Ochromonas (Bressan, et al., 1980a).

The chlorophyll a content of phytoplankton collected from the 0.1-m stratam of Lawrence Lake and Wintergreen Lake, reported as μg chlorophyll a g^{-1} fresh wt. is presented in Figure 6. No significant correlation ($p >> 0.05$) between particulate cAMP and chlorophyll a content could be determined for either Lawrence Lake (Figure 7) or Wintergreen Lake (Figure 8) on a seasonal basis. However, chlorophyll a content was significantly correlated ($r^2 = 0.98$) to particulate cAMP levels during the development of the blue-green algal association (early Aug - early Sept) in Wintergreen Lake (Figure 8 - insert). Similarly, a relationship between chlorophyll a and particulate cAMP was found during the development of the spring association of Cyclotella and cryptomonads (Figure 7 - insert), although this correlation was not significant at the 5% level, ($0.20 > P > 0.10$).

The dynamics of alkaline phosphatase in Lawrence Lake and Wintergreen Lake are presented in Figure 9, as μmoles P release 10^4 g^{-1} fresh wt min^{-1}. From these data, it appeared that Lawrence Lake plankton were much more phosphorus-limited throughout the growing season than the plankton community in Wintergreen Lake as evidenced by the fact that specific phosphatase activity was generally an order of magnitude greater in Lawrence Lake. However, phosphatase activity could not be correlated to the dynamics of particulate cAMP, either on a seasonal

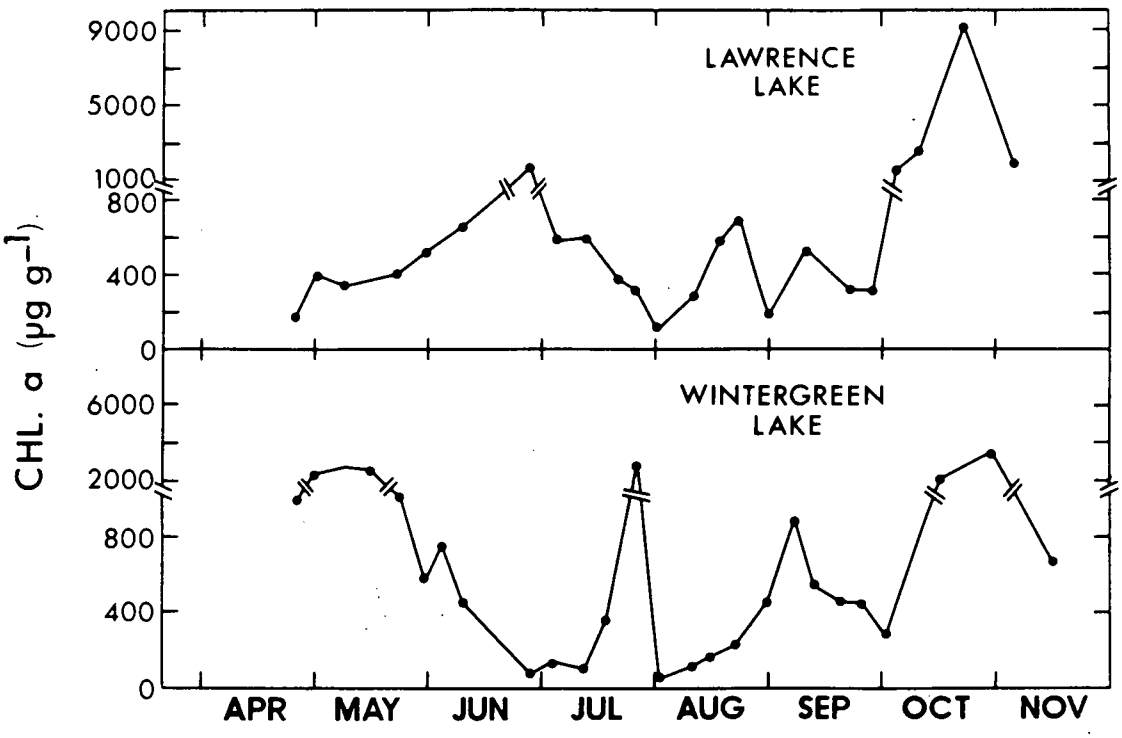

Figure 6. Chlorophyll a content (µg chlor a g^{-1} fresh wt) for epilimnetic samples from Lawrence Lake and Wintergreen Lake, 1979.

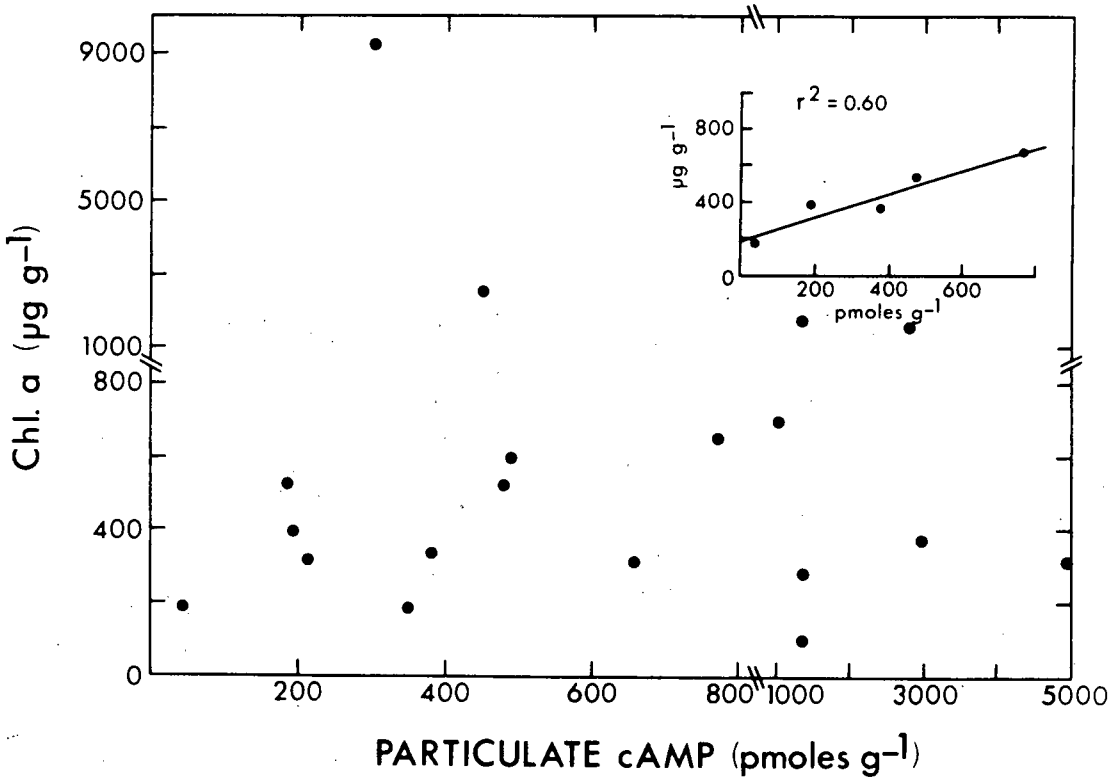

Figure 7. Correlation between particulate cAMP levels (pmoles g^{-1} fresh wt) and chlorophyll a content (µg chlor a g^{-1} fresh wt) for epilimnetic samples from Lawrence Lake, 1979. Insert demonstrates the same correlation for the development of the spring Cyclotella association (late April-late May, 1979).

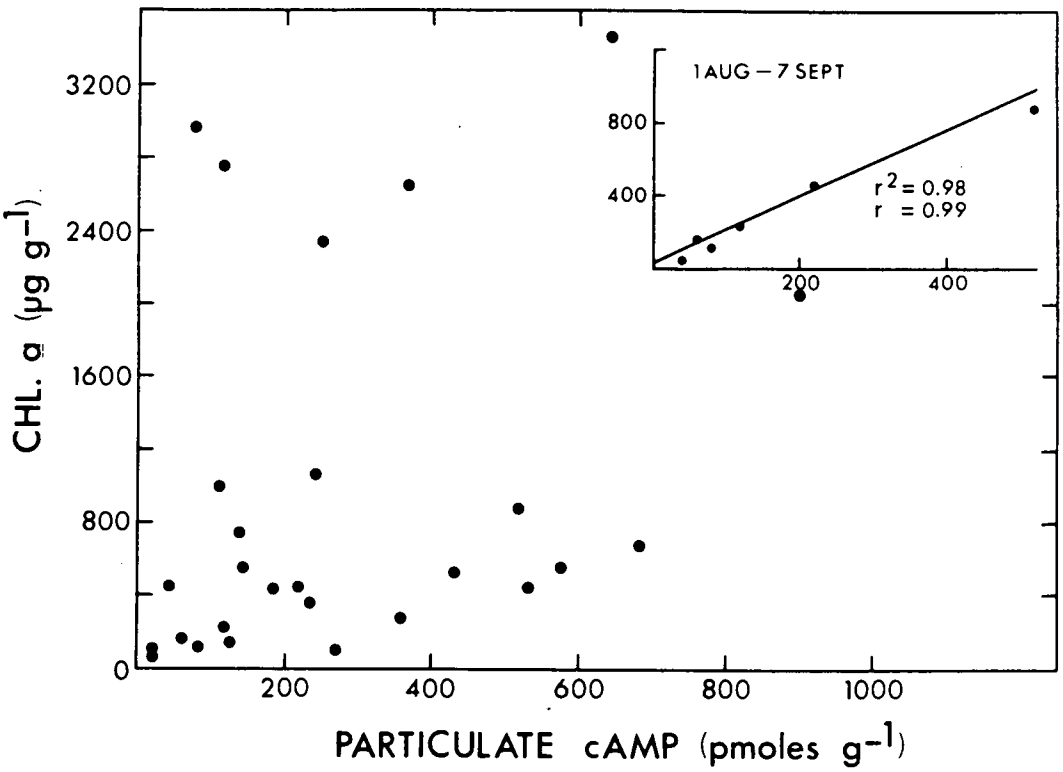

Figure 8. Correlation between particulate cAMP levels (pmoles g^{-1} fresh wt) and chlorophyll a content (μg chlor a g^{-1} fresh wt) for Wintergreen Lake, 1979. Insert demonstrates the same correlation for the late summer blue-green algal association.

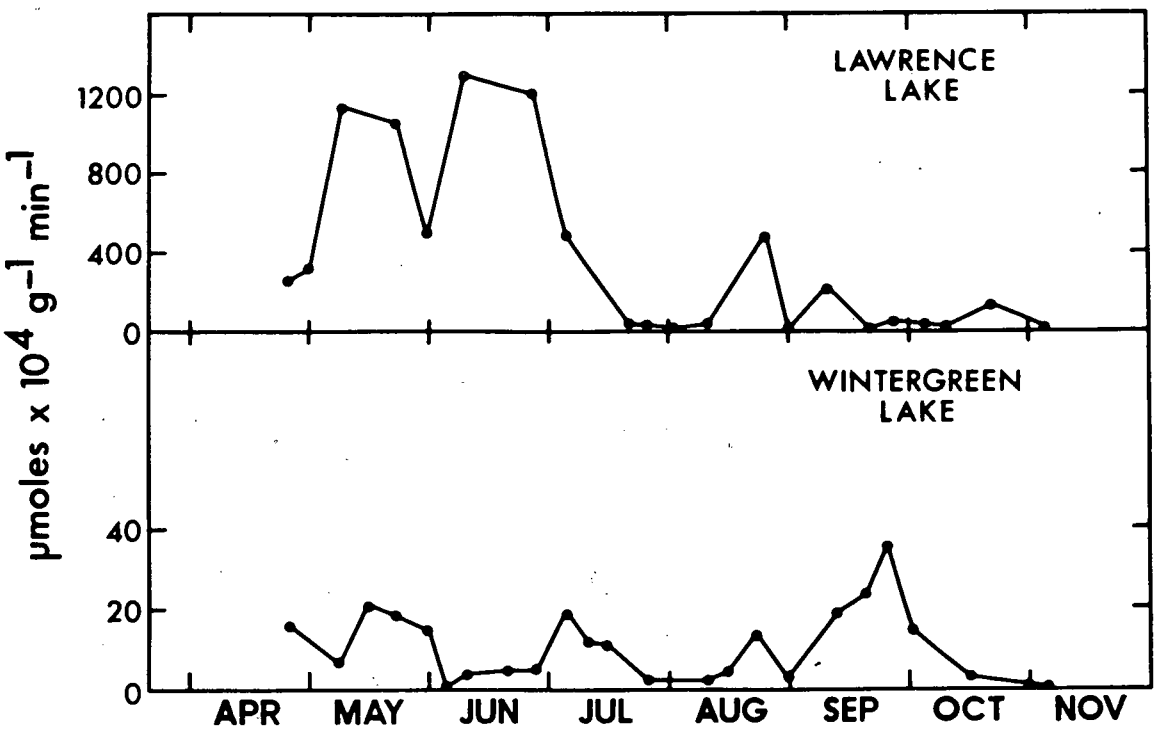

Figure 9. Alkaline phosphatase specific activity (μ moles P released × 10^4 g^{-1} fresh wt) in epilimnetic samples collected from Lawrence Lake and Wintergreen Lake, 1979.

basis or during the development of any association in Wintergreen Lake ($P >> 0.05$). In Lawrence Lake (Figure 10), phosphatase activity could not be correlated to particulate cAMP on a seasonal basis, but was significantly correlated to the increase in phosphatase activity of the spring Cyclotella association ($r^2 = 0.93$; $P < 0.05$) (Figure 10 -insert).

When the specific carbon fixation rate in situ in Lawrence Lake was compared on a seasonal basis to the dynamics of particulate cAMP, a significant correlation ($r^2 = .63$; $P < .05$) was determined for all but three sample dates (Figure 11). Points A, B and C represent data collected at the particulate cAMP maxima in the spring, midsummer, and fall periods, respectively, in Lawrence Lake, suggesting that thresholds may have existed above which cAMP no longer affected the productivity of spring, midsummer, and fall phytoplankton associations. In situ productivity measurements were not conducted on Wintergreen Lake.

All correlations previously described have dealt with the relationship between particulate cAMP and metabolic parameters in Lawrence Lake and Wintergreen Lake. In no case could dissolved cAMP be significantly correlated with chlorophyll a, in situ rates of primary productivity or alkaline phosphatase activity in either lake, on a seasonal basis or during the development of any phytoplanktonic association. The relationship of cAMP to the production of heterocysts in situ could not be investigated due to the paucity of heterocyst-forming blue-green algae in these lakes during the study period.

CONCLUSIONS

This investigation demonstrated that particulate and dissolved cAMP exist in aquatic systems in quantities similar to those reported for

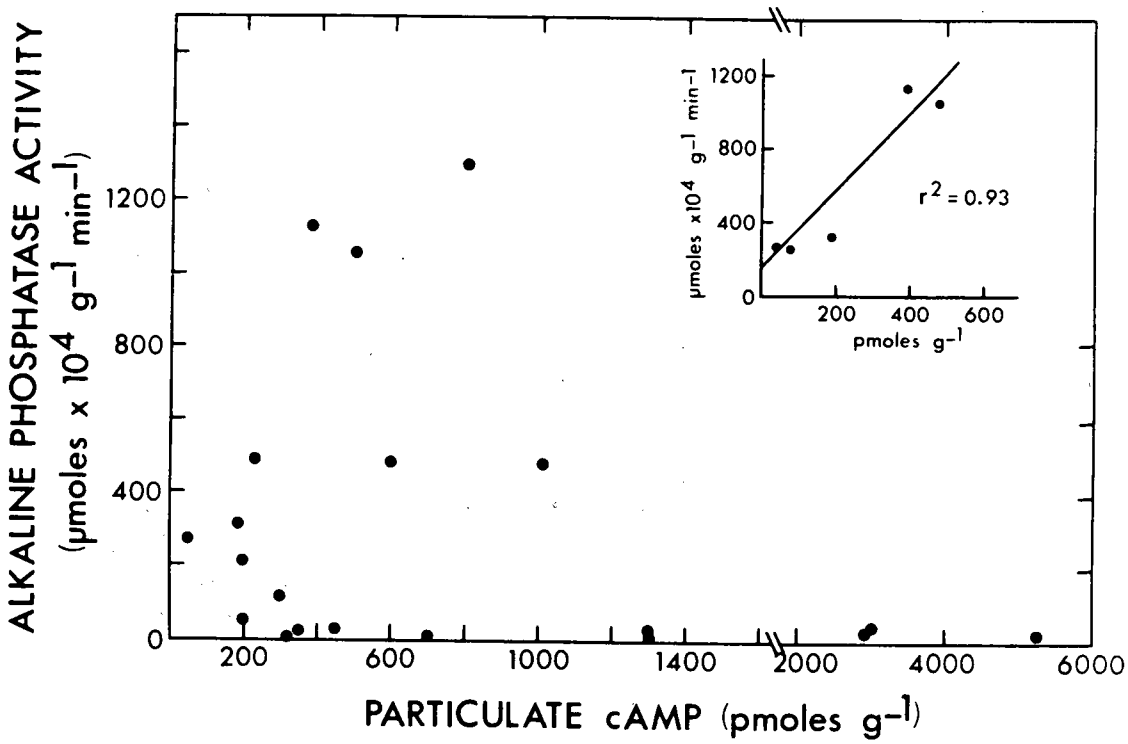

Figure 10. Correlation between particulate cAMP levels (pmoles g^{-1} fresh wt) and alkaline phosphatase specific activity (μmoles P released x 10^4 g^{-1} fresh wt) for epilimnetic samples from Lawrence Lake, 1979. Insert demonstrates the same correlation for the development of the spring Cyclotella association (late April-late May, 1979).

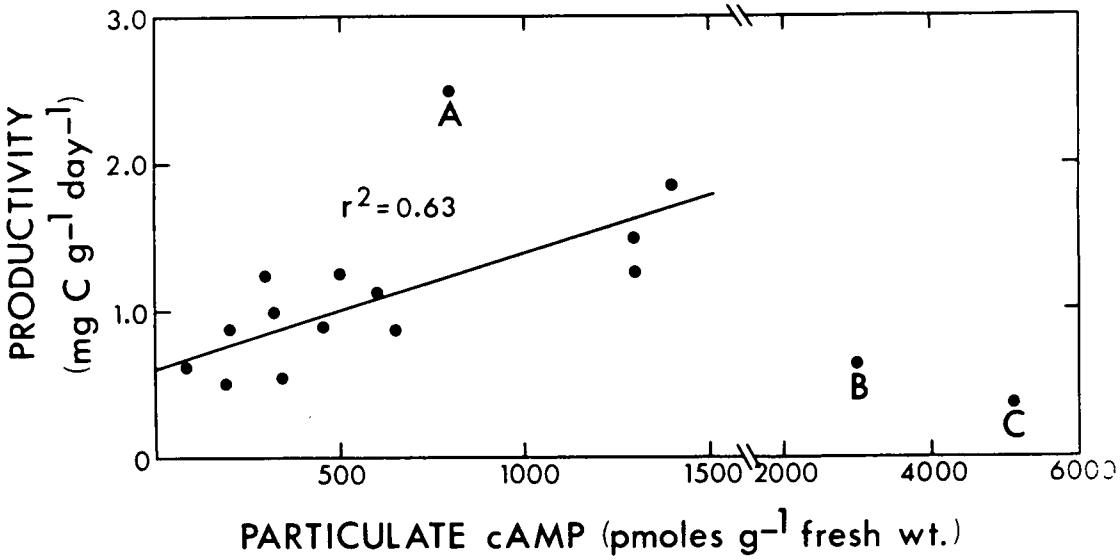

Figure 11. Correlation between particulate cAMP levels (pmoles g^{-1} fresh wt) in samples collected from the 0.1-m stratum and primary productivity (integrated values from the 0.1-m and 1-m strata in mg C fixed day^{-1} g^{-1} fresh wt) for Lawrence Lake, 1979. Points A, B, and C represent data from the spring, fall, and midsummer cAMP maxima, respectively, and were not included in the regression.

heterotrophic and photosynthetic organisms. The concentrations of both particulate and dissolved cAMP varied dynamically through the season and between lakes of differing trophic status. Significant amounts of dissolved cAMP may reach the pelagial zone of a lake through inputs from the adjacent littoral zones and from allochthonous sources.

The dynamics of cAMP in these two lakes appeared to be involved with changes in community structure throughout the growing season. Furthermore, certain phytoplankton associations may have utilized particulate cAMP as a regulator of chlorophyll synthesis, primary productivity, and alkaline phosphatase activity, although a direct functional relationship has yet to be established. Increases in extracellular cAMP through the season may have resulted from increased extracellular release of cAMP by plankton as a means of reducing intracellular cAMP levels, or by inputs from the littoral zone, but the possibility that dissolved cAMP had metabolic functions in these systems could not be discounted on the basis of these data. It was clear, however, that much of the cAMP in both lake systems occurred in the dissolved form. Previous studies on cultured phytoplankton also demonstrated that much of the cAMP present in cultures exists in the dissolved form (Bressan, et al., 1980a,b; Francko and Wetzel, 1980; Chapters I and II) but the ratio of dissolved to particulate cAMP per liter whole lakewater noted in this study was generally much higher than values reported for cultured phytoplankton.

Collectively these data indicate that cAMP may be involved in the regulation of phytoplankton population dynamics and in the regulation of certain metabolic functions in epilimnetic phytoplankton populations. The data also suggest that different phytoplankton species may utilize

cAMP to regulate different functions. The generally higher concentrations of cAMP noted through the year in oligotrophic Lawrence Lake, as opposed to hypereutrophic Wintergreen Lake, support the contention that increased cAMP production may regulate algal responses to nutrient limitation. This relationship has been demonstrated in numerous studies on cultured phytoplankton. The noted correlations between cellular cAMP levels and the rate of primary productivity and alkaline phosphatase activity in Lawrence Lake are consistent with the view that cAMP is involved in the alleviation of catabolite-repression mechanisms in phytoplanktonic organisms, similar to mechanisms known to occur in bacteria.

The physiological or ecological significance of the production and subsequent transfer of cAMP between littoral and pelagial zones of a lake, or the importance of cAMP in aquatic macrophytes could not be evaluated at this time. However, the possibility that dissolved cAMP may act as an "information" molecule in these systems cannot be discounted from these data. Thus, the transfer of cAMP between the littoral community, which often dominates the overall productivity of small lakes, and the pelagial community (or presumably vice versa), may have profound ecological consequences.

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